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FOREWORD

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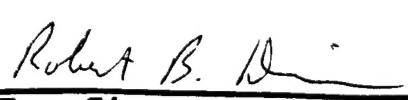
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Introduction

This project continues to focus on characterization of the potent tumorigenic interaction of c-Myc (a nuclear oncogene) and transforming growth factor α (TGF α , a growth factor) in the mammary gland. Both genes are commonly overexpressed in human breast cancer and we have established a novel bitransgenic mouse model for mechanistic studies of their co-overexpression (1). Tumors are characterized for expression of the transgenes, state of differentiation, and expression of several other malignancy-associated genes. Detailed biochemical and molecular studies are then to assess regulation of apoptosis (programmed cell death) and cell cycle aberrations as potential mechanisms of transgene interaction. Finally, xenograft transplantation of mammary cells expressing Myc are to be combined in variable proportions with cells expressing no transgene or TGF α and implanted in recipient mammary fatpads from non-transgenic or TGF α transgenic mice to assess TGF α -Myc paracrine interactions *in vivo*.

Body

The goals of the research in the second year of our project were twofold. First, we were to characterize rapid onset bitransgenic TGF α -Myc mouse mammary tumors for expression of sex steroid receptors and for indicators of epithelial differentiation status. Second, we were to characterize tumors for amplification of genes encoding TGF α , c-Myc, EGF receptor, the Myc protein partner Myn, and for expression or relevant cyclins.

Table I presents information concerning mammary tumor frequency and latency in our bitransgenic mouse model. Tumors arose in all males and virgin females containing the two transgenes with a latency of 66 days in each sex. In contrast, in virgin females, the mean tumor latency in single transgenic, Myc-bearing animals was nearly one year, and no tumors were ever seen in single transgenic TGF α -bearing animals. Table II presents the histopathologic characterization of all mammary lesions: bitransgenic tumors were adenocarcinomas. We observed that although bitransgenic mammary (and salivary) tumors arose in male and female bitransgenic animals very rapidly and independent of pregnancy, that the mammary tumors contained moderate levels of receptors for estrogen and progesterone. However, growth regulation of tumor cells *in vivo* and *in vitro* was independent of these steroids. In addition, the tumors were of epithelial morphology and expressed cytokeratins as detected with a pan-cytokeratin antibody. Tumors contained multiple copies of the expected transgenes, but were not amplified for genes encoding EGF receptor, Myn, or cyclins. These results were reported in the scientific literature (1, see Appendix).

Because rapid progress was made on these aims for Year 2, we moved on to aims for Year 3 (Task 3): to examine mRNA and protein expression in bitransgenic tumors for the same genes. Interestingly, we observed that co-overexpression of the two transgenes resulted in their expression at the mRNA level which was clearly in excess of either transgene expression in single transgenic, long latency control tumors (Figure 1). This strongly suggested a selective advantage for transgene co-overexpression. Northern blot data were confirmed by *in situ* hybridization data in mammary and salivary gland tumors. When we examined Myn and Cyclin D₁ mRNA expression, they were also observed to be highly expressed at the mRNA level consistent with a high degree of proliferation and malignant transformation of bitransgenic tumors. The EGF receptor (Figure 2) was expressed at moderate levels in bitransgenic and single transgenic tumors (1, see Appendix).

We were able to establish cell lines from the single and bitransgenic tumors, in order to further address mechanisms of interaction. We observed that compared to single transgenic tumor cells, the bitransgenic tumor cells proliferated more rapidly and more readily grew in anchorage independent colonies. In addition, c-Myc single transgenic tumor cells exhibited a high degree of apoptosis (programmed cell death) compared to TGF α -expressing single and bitransgenic tumor lines. This is illustrated with an ELISA-apoptosis assay (Boehringer Mannheim) in Figure 3, where the presence

or absence of the Myc transgene suppresses apoptosis to the assay background level. Apoptosis was suppressed *in vitro* in c-Myc cells by addition of TGF α , EGF, FGF-2, and IGF-1; the EGF receptor-mediated effects were selectively blocked with an EGF receptor-selective tyrosine kinase inhibitor (Figure 4). Finally, we confirmed the high level of c-Myc-associated apoptosis *in vivo*, selectively in single transgenic tumors using several assays, including an *in situ* nick end labelling assay. These results have just been published (2, see Appendix). As a followup, we observed that TGF α induction of the survival factor induced Bcl-X_L was associated with suppression of apoptosis in c-Myc-overexpressing mouse mammary tumor cells *in vitro* and *in vivo* (3).

A continuing goal of the study is to evaluate paracrine interactions of the two transgenes (Task 1b) In the first year of our work, we encountered a probable immunologic barrier to our cross-transplantation experiments due to different backgrounds of Myc and TGF α transgenic mice. However, Charles River breeders has now backcrossed their Myc mice into the FVB strain (identical to the TGF α background). Using skin grafts, we have now confirmed that myc tissue is compatable when transplanted to the TGF α strain and *vice versa*. We now expect to complete these paracrine transplantation experiments in Year 3.

Additional progress was made on studies closely related to our specific aims and acknowledging support of the PI by this grant. We observed that Myc and TGF α single transgenic tumors express aberrantly processed forms of the EGF family member amphiregulin (4), and that amphiregulin was capable of causing preneoplastic growth of the mouse mammary gland (5). In addition, we observed that in analogy to TGF α , amphiregulin is inducable in human breast cancer cells, by both estrogen and by phorbol ester activators of protein kinase C (6). We also collaborated with another laboratory using our TGF α transgenic mice and observed that these mice exhibited preneoplastic-appearing female reproductive tract lesions. However, these lesions were non-progressing; DES induced fully malignant lesions and positively interacted in this respect with TGF α (7). Finally, we published three review articles on the subject of this grant (8-10).

Table 1 Number of offspring in each of four genotype groups

Mean mammary gland tumor onset times and tumor frequency in each group is presented.

Genotype	No. of animals (%) ^a	Mean tumor onset time (days)	Tumor incidence
TGF α /c-myc	23 (15)	66 \pm 12	100% ^b
TGF α	24 (15)	NA	0%
c-myc	39 (25)	298 \pm 55	50%
Wild type	71 (45)	NA	0%

^a Total offspring number is 157.

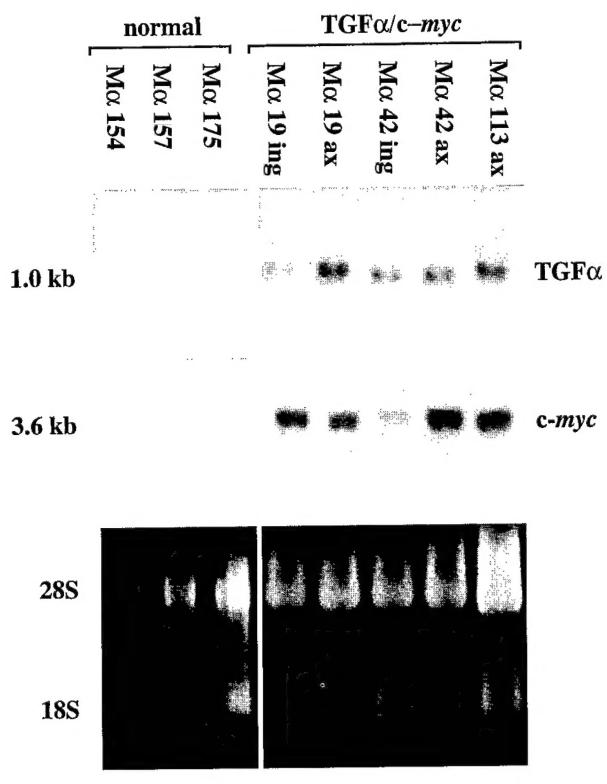
^b One hundred % equals 20/20 animals; 3 animals died at very young ages. NA, not available (tumors were not observed for more than 1 year).

Table 2 Summary of histopathological findings in mammary glands of transgenic mice at 3 and 7 months of age

Genotype	3 mos.	7 mos.
TGF α /c-myc	Multiple adenocarcinomas (types A and B) in females and males ^a	NA
TGF α	Normal	Cystic ducts
c-myc	Normal	Atypical hyperplasia to adenocarcinoma
Wild type	Normal	Cystic ducts

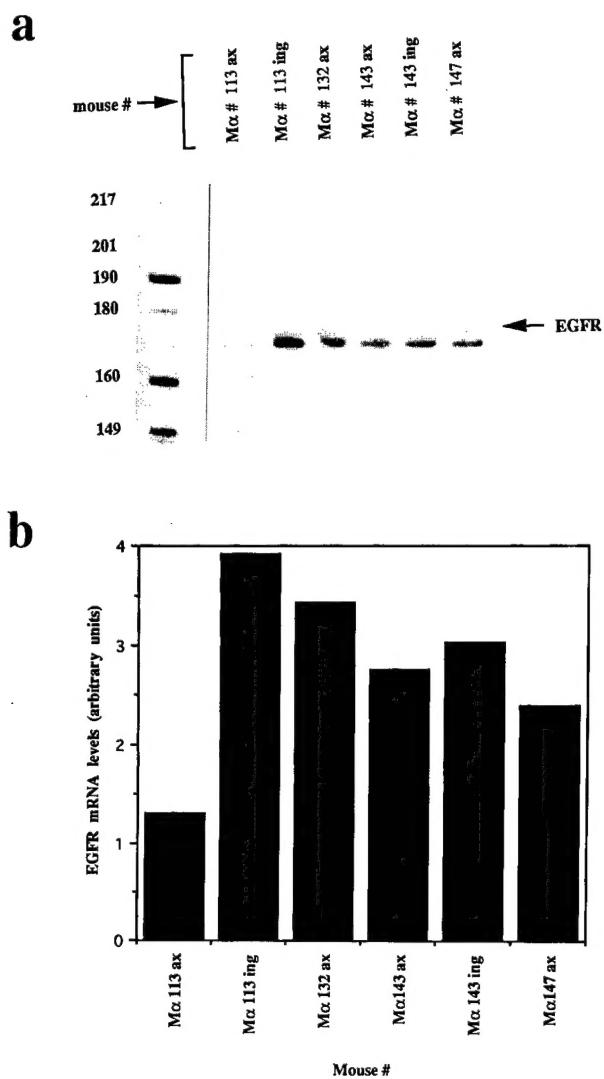
^a Histopathology from both virgin females and males. All other diagnoses are from virgin females only. NA, not available (all animals of this genotype are deceased at this time point).

Figure 1



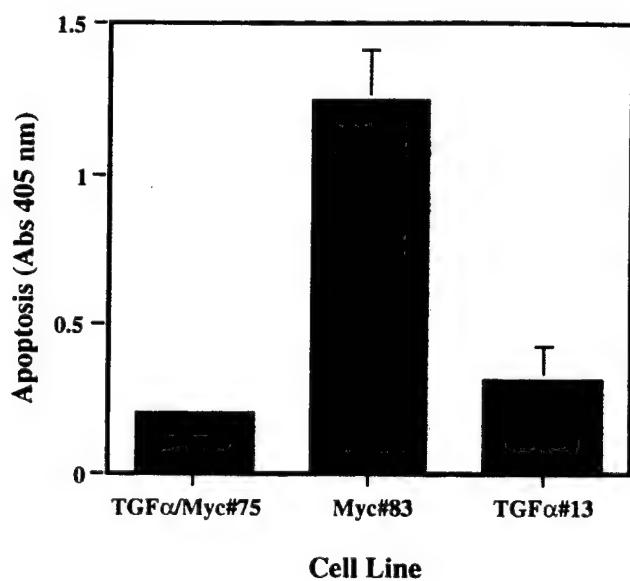
Northern analysis showing expression of the TGF α and *c-myc* transgenes in mammary gland tumors from double transgenic animals and in normal glands from single transgenic and wild-type animals. The TGF α transcript is 1.0 kb, and the *c-myc* transcript is 3.6 kb. Note that the endogenous 2.3-kb *c-myc* transcript is not seen here. Loading controls are the 18S and 28S ribosomal RNA bands. M α 19 through 175 denotes the number of each animal used here. Ax, axillary gland tumor; ing, inguinal gland tumor.

Figure 2



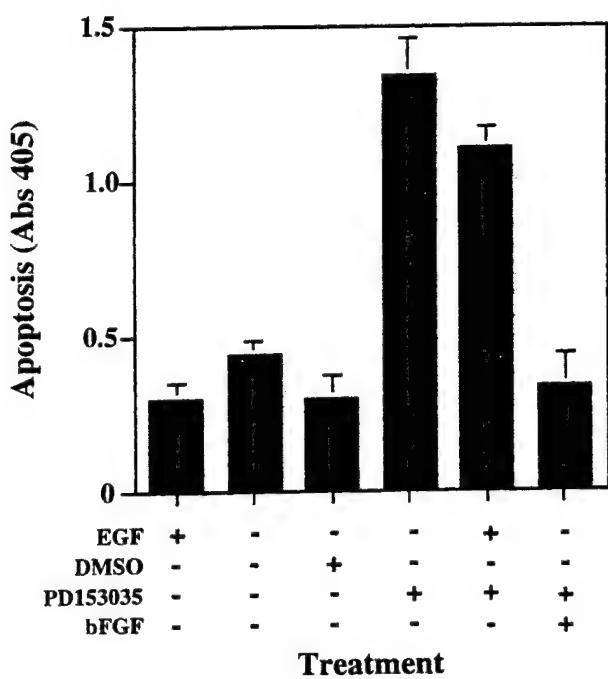
Expression of the endogenous EGFR mRNA measured by RNase protection assay. RNA levels were compared between mammary gland tumors from double transgenic animals. *b*, a scanned version of the data in *a*. M α 113 through M α 147 denotes the number of each double transgenic mouse used in the assay. *Ax*, axillary gland tumor; *ing*, inguinal gland tumor.

Figure 3



Detection of apoptosis in tumor-derived cell lines by an ELISA-apoptosis assay. Apoptotic DNA fragments were detected in cytoplasmic lysates via a histone-DNA ELISA. Peroxidase substrate conversion was quantitated by measuring absorbance at 405 nm.

Figure 4



Induction of apoptosis in the TGF α Myc=75 line by PD153035, a specific inhibitor of EGF receptor tyrosine kinase activity from Parke Davis Corp. Cells were incubated for 24h with the indicated additions (10ng/ml EGF or bFGF, 10 μ M PD153035, 1 μ l/ml DMSO as a control and apoptosis was measured via ELISA assay. n=4 (+ SE)

Conclusions

1. Bitransgenic TGF α /Myc tumors are derived from mammary epithelial cells and contain estrogen, progesterone, and EGF receptors.
2. While multiple copies of the two transgenes were detected, other related growth control genes were not amplified in the bitransgenic tumors.
3. Bitransgenic tumors provide a strong selection for further overexpression at the mRNA level of transgenes and expression of high levels of Myn and Cyclin D₁.
4. The mechanisms of the tumorigenic interaction of TGF α and Myc included cooperative stimulation of proliferation, anchorage independent colony formation, and suppression of apoptosis.
5. One aspect of TGF α -suppression of apoptosis involved induction of the survival-promoting Bcl-X_L gene.
6. Transgenic tumors also expressed the TGF α -family growth factor amphiregulin, but in a variety of unusual isoforms.
7. Amphiregulin, like TGF α , was capable of induction of preneoplastic outgrowth of the mouse mammary gland.
8. Amphiregulin mRNA and protein synthesis are upregulated by estrogen and by protein kinase C in human breast cancer cells, in analogy to the previously-described regulation of TGF α .
9. Female TGF α transgenic mice are susceptible to reproductive tract abnormalities and to DES-induced neoplasias, in addition to Myc-induced mammary tumors.
10. In summary, these results strongly support the initial premises of the grant; that the Myc transcription factor and the TGF α family of growth factors are each potent modulators of mammary tumor onset and progression. Binary interaction of Myc and TGF α in the mouse mammary gland allows rapid tumor development due to a synergistic, multifactorial fashion.

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Appendix

Amundadottir, L.T., Johnson, M.D., Merlino, G.T., Smith, G.H., and Dickson, R.B.: Synergistic interaction of transforming growth factor α and c-myc in mouse mammary and salivary gland tumorigenesis. Cell Growth and Different, 6:737 -748 1995.

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Synergistic Interaction of Transforming Growth Factor α and c-myc in Mouse Mammary and Salivary Gland Tumorigenesis¹

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Abstract

The c-myc oncogene is commonly amplified in breast cancer and is known to interact synergistically with transforming growth factor α (TGF α) *in vitro* to promote phenotypic transformation of mammary epithelial cells. In addition, both genes are under sex steroid hormone regulation in breast cancer. We have used a bitransgenic mouse approach to test the relevance of Myc-TGF α interaction in mammary gland tumorigenesis of virgin animals *in vivo*. We mated single transgenic TGF α and c-myc mouse strains to yield double transgenic offspring for TGF α and c-myc. All (20 of 20) double transgenic TGF α /c-myc animals developed synchronous mammary tumors at a mean age of 66 days. An unexpected finding was that tumor latency and frequency in males and virgin females were identical. Thus, two gene products that are known to be coinduced in breast cancer by the sex hormones estrogen and progesterone strongly synergize to induce synchronous mammary tumors, independent of sex. The tumors, despite being estrogen receptor positive, were readily transplanted as highly malignant s.c. cancers in ovariectomized nude mice. Although approximately one-half of single transgenic c-myc virgin females also eventually developed mammary gland tumors, these were stochastic and arose after a long latency period of 9–12 months. Single transgenic virgin TGF α females and males, c-myc males, and transgene-negative littermates did not develop tumors (ages up to 15 months).

The salivary glands of double transgenic animals also coexpress the two transgenes and show pathological abnormalities ranging from hyperplasias to frank adenocarcinomas. In contrast, the salivary glands of single transgenic and wild-type animals showed only mild hyperplasias or metaplasias, but tumors were not observed.

In situ hybridization analysis of mammary and salivary glands revealed that hyperplastic and tumorous areas colocalize with regions that overexpress both the TGF α

and c-myc transgenes. This indicates that there is a requirement for the presence of both proteins for transformation of these glands. In summary, TGF α and c-Myc synergize in an extremely powerful way to cause breast and salivary gland tumorigenesis in males and virgin females without a requirement for pregnancies.

Introduction

Gene amplification and/or deregulated expression of a number of genes are frequent findings in human breast cancer. Among these are the genes for c-myc and TGF α .³ The protein product of the c-myc gene is a nuclear phosphoprotein involved in transcriptional regulation, and TGF α is a member of the EGF family of mitogens, which bind to and activate the EGF receptor (1, 2). The c-myc proto-oncogene is amplified in 25 to 30% of breast cancer cases and is overexpressed (without gene amplification) in many more (3–6). Furthermore, amplification of the c-myc gene has been shown to correlate with poor prognosis of the disease (3, 7, 8). Although the TGF α gene is not found amplified in human breast cancer, its expression (and that of other EGF family members) is frequently increased compared to the normal gland (9–12). The EGFR is also found expressed in about 30–50% of human breast cancers with high expression associated with poor prognosis and high a degree of invasiveness (13).

Expression of both genes is induced during estrogen and progesterone treatment of hormone-responsive breast cancer cells *in vitro* (10, 14–18). In addition, treatment with antisense oligonucleotides to either TGF α or c-myc inhibits estrogen-induced expression of these genes and estrogen-stimulated growth *in vitro*, indicating that they are important mediators of estrogenic effects on cell growth (19, 20).

Transgenic mouse models have provided insight into the roles of both genes in mammary gland development and malignant progression *in vivo*. Overexpression of TGF α in the mammary gland from the mouse metallothionein promoter or the MMTV promoter/enhancer caused the appearance of mammary carcinomas after a relatively long latency period of 7–12 months. Tumors were stochastic and arose predominantly in female mice that had undergone multiple pregnancies (21–23). Transgenic mice with MMTV-myc constructs directing expression to the mammary gland also develop clonal tumors after a long latency period of 7–14 months, again with a requirement for multiple pregnancies (24).

Long latency times in transgenic mice are consistent with the hypothesis that oncogenesis is a multistage process composed of a series of genetic events (25, 26). Thus, although one proto-oncogene is overexpressed in a given

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³ The abbreviations used are: TGF α , transforming growth factor α ; EGF, epidermal growth factor; EGFR, EGF receptor; MMTV, mouse mammary tumor virus; MT, metallothionein; LTR, long terminal repeat.

organ of a transgenic mouse, the occurrence of additional events is necessary before cancer can arise. We chose to study the interaction of TGF α with c-myc, since *in vitro* studies had suggested possible cooperative interactions. In various cell types *in vitro*, overexpression of c-myc resulted in an increased responsiveness to the effects of mitogenic growth factors. For example, rodent fibroblasts and human and mouse mammary epithelial cell lines transfected with myc constructs showed transformed behavior, in many cases, only in the presence of TGF α or fibroblast growth factor family members (27–30). High levels of myc expression may, therefore, permit a tumorigenic transformation by a TGF α /EGFR autocrine growth mechanism, or it may sensitize cells to such a mechanism.

The interaction of TGF α and c-Myc has not been studied in mammary gland transformation *in vivo*, but two recent studies investigated their interaction in the liver and pancreas with bitransgenic mouse models. Animals expressing both transgenes from liver- and pancreatic-specific promoters formed tumors in these organs at an elevated rate compared to single transgenic animals, suggesting a synergistic interaction (31, 32).

We show here that TGF α and c-Myc cooperate in an extremely powerful, synergistic manner in mouse mammary and salivary gland tumorigenesis. Double transgenic male and virgin female mice develop synchronous mammary tumors in all glands at about 2 months of age, whereas single transgenic animals develop clonal tumors at about 12 months of age or not at all. In addition, epithelial rudiments from 3-week-old TGF α /c-myc double transgenic animals could be established in nude mice, indicating that the mammary gland is transformed right at the start of its development. Tumors were also found in salivary glands of double transgenic animals at 3 months of age, whereas single transgenic and wild-type mice have not been observed to develop tumors. Expression of the TGF α and c-myc transgenes was associated with hyperplastic and tumorous areas in mammary and salivary glands, indicating a requirement for the presence of both gene products for malignant transformation.

Results

Generation of TGF α /c-myc Double Transgenic Mice. Heterozygous mice transgenic for TGF α (MT-TGF α MT100 strain) (21) and c-myc (MMTV-c-myc M strain) (24) were mated to yield offspring of four possible groups: double transgenic TGF α /c-myc mice; single transgenic TGF α mice; single transgenic c-myc mice; and mice negative for transgenes (wild type). Since the parental mice are of FVB/N (TGF α) and CD-1 × C57BL/6J (c-myc) backgrounds, offspring mice of all groups are of the following genetic background: FVB/N/CD-1 × C57BL/6J. Mice of the two single transgenic groups and of the wild-type group serve as controls in a similar genetic background as the double transgenic TGF α /c-myc mice.

At 3 weeks of age, offspring were weaned, and DNA was extracted from tail biopsies. Screening for transgenes was performed by Southern blot analysis and/or PCR (data not shown). According to Mendelian rules, when mating animals heterozygous for two traits, 25% of offspring should fall into each of four possible genotype groups. However, of 157 offspring, 45% were wild type; 15 and 25% were single transgenic for TGF α and c-myc, respectively; and 15% were double transgenic TGF α /c-myc (Table 1). Thus, there

Table 1 Number of offspring in each of four genotype groups

Mean mammary gland tumor onset times and tumor frequency in each group is presented.

Genotype	No. of animals (%) ^a	Mean tumor onset time (days)	Tumor incidence
TGF α /c-myc	23 (15)	66 ± 12	100% ^b
TGF α	24 (15)	NA	0%
c-myc	39 (25)	298 ± 55	50%
Wild type	71 (45)	NA	0%

^a Total offspring number is 157.

^b One hundred % equals 20/20 animals; 3 animals died at very young ages. NA, not available (tumors were not observed for more than 1 year).

Table 2 Summary of histopathological findings in mammary glands of transgenic mice at 3 and 7 months of age

Genotype	3 mos.	7 mos.
TGF α /c-myc	Multiple adenocarcinomas (types A and B) in females and males ^a	NA
TGF α	Normal	Cystic ducts
c-myc	Normal	Atypical hyperplasia to adenocarcinoma
Wild type	Normal	Cystic ducts

^a Histopathology from both virgin females and males. All other diagnoses are from virgin females only. NA, not available (all animals of this genotype are deceased at this time point).

appeared to be a selection bias against mice positive for the TGF α transgene. A reduced body weight at weaning was not associated with the lower frequency genotypes (data not shown), in contrast to observations of Luetteke *et al.* (33) for a different strain of MT-TGF α transgenic mice. However, we have noted that TGF α -positive mice consistently die at younger ages than c-myc single transgenic and wild-type mice and show signs of malnutrition. This is probably due to the effects of the TGF α transgene product on the stomach, as described previously (34). Offspring of each genotype group were approximately equally divided between females and males.

Synergistic Induction of Mammary Gland Tumors in TGF α /c-myc Double Transgenic Mice. Of 23 double transgenic TGF α /c-myc animals, 20 developed multiple mammary tumors at a mean age of 66 ± 12 days, and three mice died from other causes at very young ages. We can, therefore, conclude that all mice of the TGF α /c-myc genotype that reached an age of about 2 months developed mammary gland cancers. An additional striking finding was that tumors arose in both virgin female and male animals with the same latency and frequency. Frank tumors (*i.e.*, palpable) arose first in axillary mammary glands (glands nos. 1, 2, and 3) and then subsequently in inguinal glands (nos. 4 and 5). The average number of palpable tumors at time of necropsy were 2.5/mouse. In addition, pathological diagnoses of hematoxylin/eosin-stained sections revealed the presence of adenocarcinomas in glands without frank palpable tumors, thus showing that every mammary gland from double transgenic animals was cancerous. Surprisingly, no normal tissue was found adjacent to mammary gland tumors in double transgenic animals; therefore, the whole gland could be characterized as malignant. Even a very young (5 weeks old) TGF α /c-myc-positive female was diagnosed as having

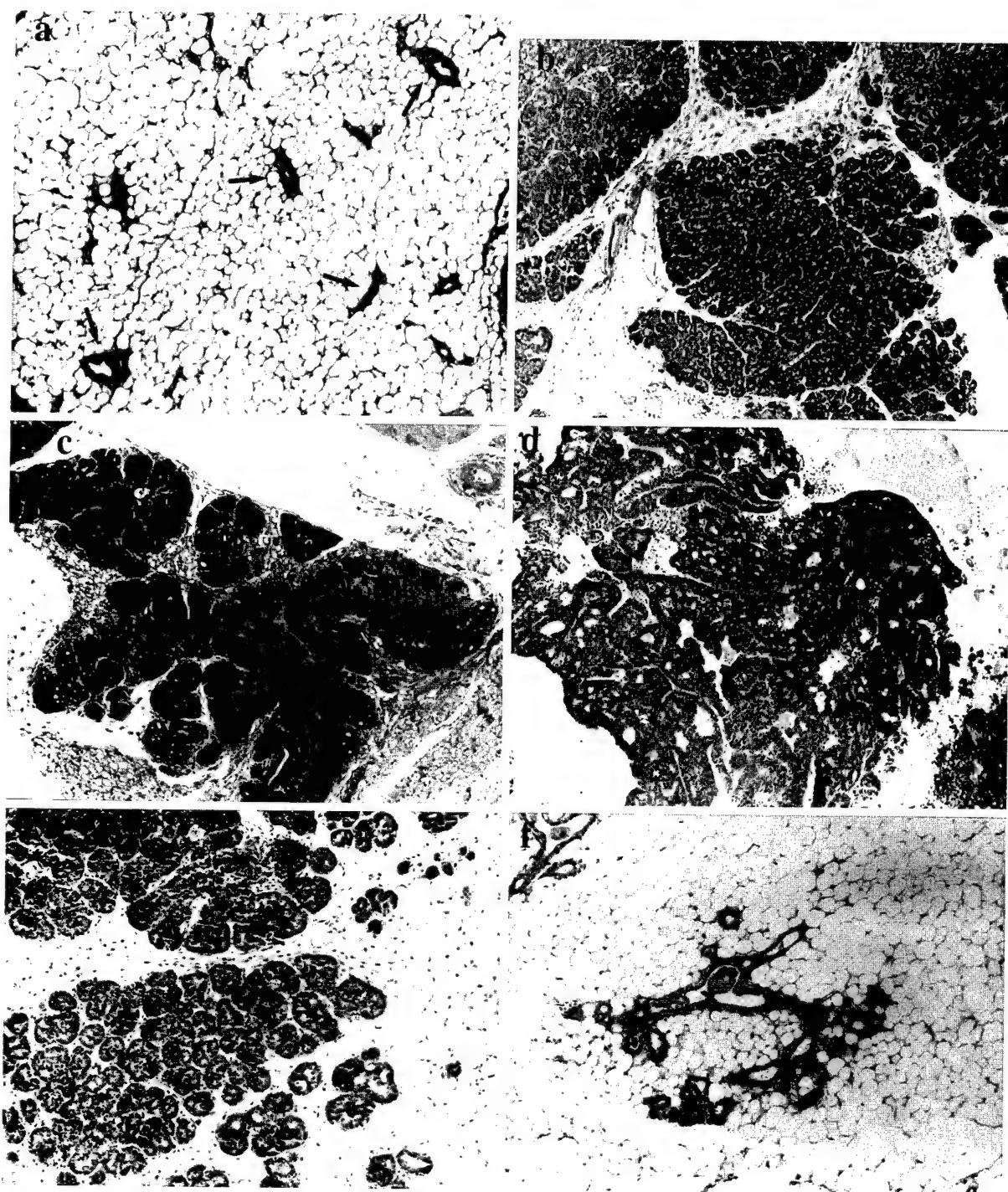


Fig. 1. Hematoxylin & eosin-stained sections of mammary glands. *a*, a normal virgin gland at 3 months of age. It is representative of TGF α and *c-myc* single transgenic animals and of nontransgenic littermates at this age. Arrows, epithelial ducts. Panels *b-d*, mammary gland tumors from double transgenic TGF α /*c-myc* animals at 3 months of age. *e*, a section of an inguinal mammary gland from a 5-week-old TGF α /*c-myc* female. *f*, an atypical hyperplastic gland from a *c-myc* single transgenic virgin female at 9 months of age. $\times 100$.

mammary gland adenocarcinoma based on histopathology. This is striking since, at this age, the gland is not fully developed. Our results suggest that, in this model, overexpression of TGF α and *c-myc* is sufficient to cause a com-

plete tumorigenic transformation of the mouse mammary gland.

Mean tumor onset times and frequency are shown in Table 1. Pathological diagnosis of mammary glands from



Fig. 2. Whole-mount staining of mammary glands from a TGF α /c-myc animal (a) and from a wild-type animal (b). The carmine alum stain reveals the epithelial network of the gland and the lymph node (In). The animal in a was 24 days old, and the one in b was 28 days old. Note tumorous nodules (n) in the mammary gland from the TGF α /c-myc animal (a) and normal ductal pattern in the wild-type animal (b).

transgenic mice of each genotype group, at 3 and 7 months of age, is shown in Table 2. Representative hematoxylin/eosin-stained sections are shown in Fig. 1. The normal virgin gland at 3 months of age is mostly composed of adipose tissue, with scattered epithelial ducts consisting of two layers of cells (Fig. 1a). It is representative of pathology from the two single transgenic groups and the wild-type group at 3 months of age. In contrast, every mammary gland from double transgenic animals (virgin females and males) is tumorous at the same age (Fig. 1, b-d). Tumors were classified as adenocarcinomas of types A and B. A type tumors are fairly well differentiated, with the acinar struc-

ture of the gland prominent and two layers of epithelial cells seen surrounding lumens (Fig. 1b). Type B is less organized and locally invasive (Fig. 1d). When tumor sections were stained with periodic acid-Schiff stain, the basement membrane was seen intact in type A tumors but was often disrupted in type B tumors (data not shown). No distant metastases have been found to date. Fig. 1e shows adenocarcinoma from a 5-week-old double transgenic virgin female animal. At this age, the epithelial tree has not fully penetrated the mammary fat pad.

About 50% single transgenic *c-myc* virgin females also developed mammary gland tumors, but these were stochas-

tic and arose only after a very long latency period of 298 ± 55 days. The remainder had mild atypical hyperplasias and cystic ducts (Fig. 1f). Single transgenic virgin TGF α mice and transgene-negative littermates have not developed tumors to this date (ages up to 15 months). In the case of single transgenic males, we observed atypical hyperplastic areas in mammary glands of a 14-month-old single transgenic c-myc male (data not shown). Mammary glands from single transgenic TGF α males and wild-type males at the same ages were normal.

A whole organ staining (termed whole-mount staining) of mammary glands from virgin double transgenic animals at 24 days of age revealed multiple nodules in each gland that appeared tumorous (Fig. 2a). These were successfully established in nude mice, indicating that the gland is transformed from the start of its development. In comparison, whole-mount staining of mammary glands from wild-type virgin animals at 28 days of age revealed only the normal ductal pattern (Fig. 2b).

The observation that mammary gland tumors arose in double transgenic TGF α /c-myc males as well as in virgin females suggested that they might be estrogen independent. Estrogen receptor ligand binding assays revealed that tumors from males and females contained from 13–30 fmol/mg protein of the receptor, and are, therefore, considered estrogen receptor positive (data not shown). Control tumors (MCF-7 or MKL-4 cells grown as tumors in nude mice) contained about 3-fold higher levels of receptor. The ovariectomizing of TGF α /c-myc females ($n = 2$) at the time of weaning did not result in a significantly delayed tumor onset (69 versus 66 days). In addition, both axillary and inguinal mammary gland tumors could be successfully transplanted into ovariectomized nude mice (data not shown). Together, these data indicate that, although relatively low levels of the estrogen receptor are present in mammary gland tumors as measured by binding to ligand, they are not dependent on estrogen for growth.

Expression of Transgenes and the Epidermal Growth Factor Receptor in Mammary Gland Tumors. We have used Northern analysis, RNase protection assays, *in situ* hybridization analysis, and immunohistochemistry to examine the expression of transgenes and that of the EGFR gene in mammary gland tumors from double transgenic TGF α /c-myc animals. RNA expression was compared between axillary mammary gland tumors (frank tumors or lumps) and inguinal gland tumors (carcinoma revealed by histopathology) of TGF α /c-myc animals.

Transgenes were expressed in all mammary gland tumors from double transgenic animals but were not detectable in normal glands from single transgenic animals at 3 months of age (Fig. 3). There was about a 5-fold difference in the expression of the c-myc transgene, and about 7-fold for the TGF α transgene between the lowest- and highest-expressing tumor. An association of transgene expression at the RNA level and pathological diagnosis (adenocarcinoma type A versus B), location (axillary versus inguinal glands), tumor size, or sex was not observed. Expression of TGF α and c-myc was not detected in normal glands by this method. Transgene expression was also examined by *in situ* hybridization analysis to establish the pattern of transgene expression in the tumors (Fig. 4, a, c, and e). Sequential tumor sections from double transgenic TGF α /c-myc animals were hybridized to 35 S-labeled TGF α and c-myc probes. We observed a very strong and uniform expression of c-myc mRNA in mammary gland tumors from double

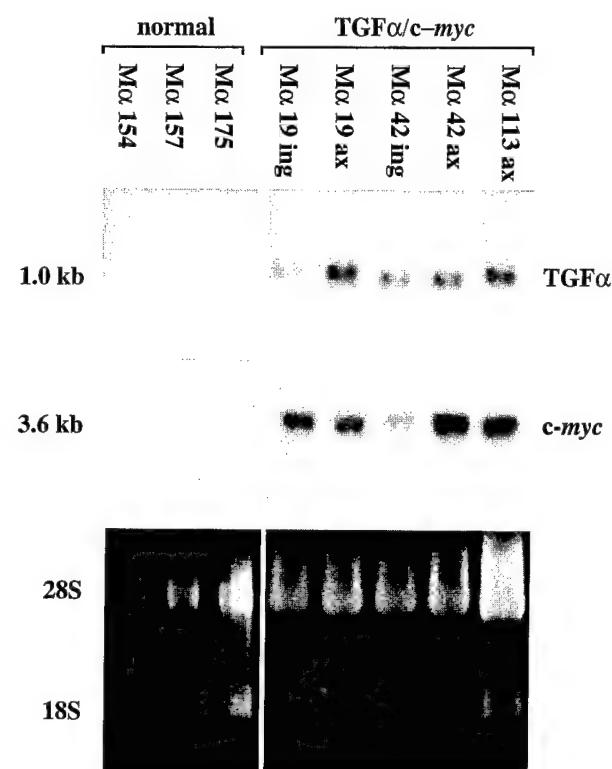


Fig. 3. Northern analysis showing expression of the TGF α and c-myc transgenes in mammary gland tumors from double transgenic animals and in normal glands from single transgenic and wild-type animals. The TGF α transcript is 1.0 kb, and the c-myc transcript is 3.6 kb. Note that the endogenous 2.3-kb c-myc transcript is not seen here. Loading controls are the 18S and 28S ribosomal RNA bands. Mx 19 through 175 denotes the number of each animal used here. Ax, axillary gland tumor; ing, inguinal gland tumor.

transgenic animals at 3 weeks of age and higher. A scattered expression of TGF α mRNA was noted in most areas of the mammary glands by this method. An immunohistochemical evaluation of TGF α protein levels in bitransgenic tumors with a TGF α -specific antibody also revealed a scattered pattern of strong expression but no association of enhanced staining with a more aggressive phenotype (Fig. 5, a and b). We also measured endogenous EGFR mRNA levels in tumors from double transgenic animals by RNase protection assays. As seen in Fig. 6, EGFR mRNA levels were comparable in all but one mammary gland tumor (axillary tumor from TGF α /c-myc animal no. 113). An association of EGFR mRNA levels with pathological diagnosis, location, tumor size, or sex was not seen.

Synergistic Induction of Salivary Gland Tumors by TGF α and c-myc. The MT promoter is active in most epithelial tissues, whereas the MMTV promoter is restricted to only a few tissues. Therefore, the MMTV promoter limits coexpression of the transgenes to mammary glands, salivary glands, and some reproductive organs. An interaction between TGF α and c-myc was not observed in reproductive organs, but a positive interaction was noted in the salivary glands. Ductule hyperplasia (sometimes with atypia) was seen in all salivary glands of double transgenic TGF α /c-myc virgin female and male animals at 3 months of age. In some cases, squamous metaplasia was observed in the sublingual gland,

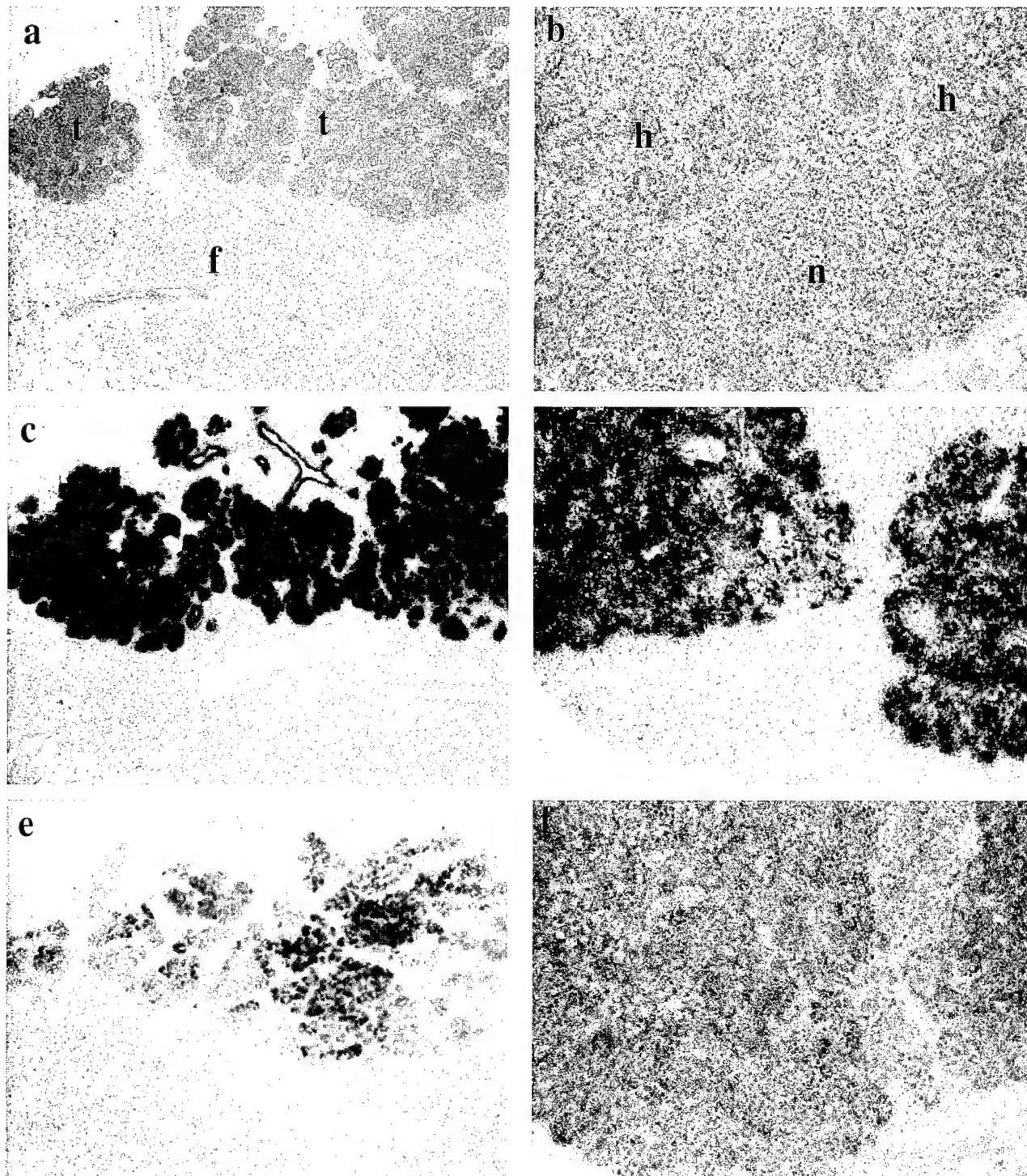


Fig. 4. *In situ* hybridization analysis of mammary and salivary glands from TGF α /c-myc animals. Sequential paraffin-embedded tissue sections were hybridized to ^{35}S -labeled riboprobes generated against the TGF α and c-myc transgenes. *a* and *b*, control sections hybridized to sense riboprobes. *c* and *d*, sections hybridized to c-myc antisense riboprobes. *e* and *f*, sections hybridized to TGF α antisense riboprobes. *a*, *c*, and *e*, from mammary glands; *b*, *d*, and *f*, from salivary glands. Note a near uniform expression of the transgenes in a mammary gland tumor from this 3-week-old double transgenic animal. Observe a patchy expression of c-myc and scattered distribution of TGF α in this 6-week-old double transgenic animal. Also note that where both transgenes are expressed, the glands appear hyperplastic and tumorous. *t*, tumor; *f*, fatty tissue; *h*, hyperplastic; *n*, normal. All panels are brightfield photographs. *a*, *c*, and *e*, $\times 50$; *b*, *d*, and *f*, $\times 200$.

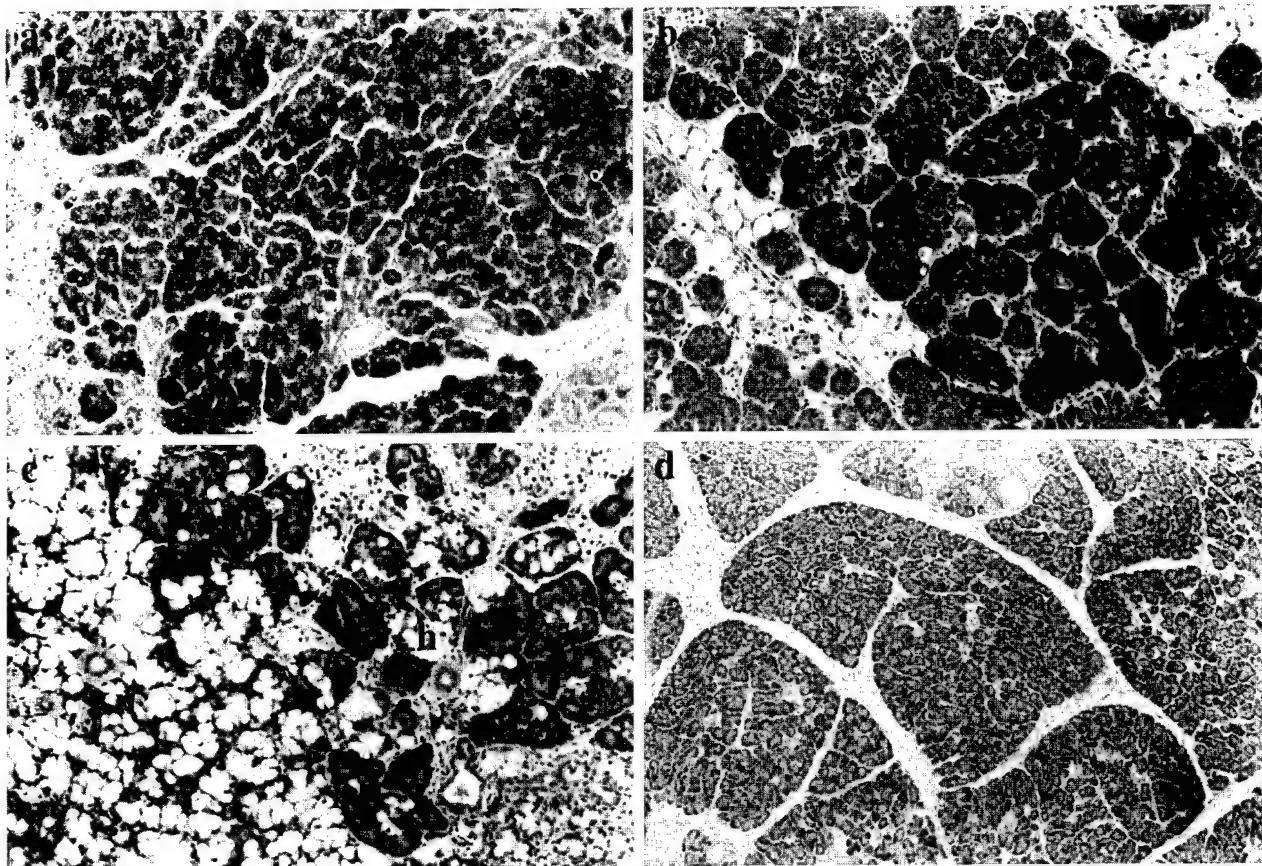


Fig. 5. Immunohistochemical staining of mammary and salivary glands from TGF α /c-myc double transgenic animals with an anti-TGF α polyclonal antiserum. *a*, a mammary gland tumor from a 3-month-old animal and *b*, from a 5-week-old animal. Both have a scattered pattern of strong TGF α staining. *c*, an immunohistochemical staining of a bitransgenic salivary gland (sublingual) with a premalignant atypical hyperplastic lesion (*h*) that stains strongly for TGF α , while the surrounding normal areas (*n*) appear negative. *d*, a negative control without a primary antibody.

and adenoma and adenocarcinoma in the parotid gland at the same age. Salivary glands of single transgenic TGF α animals showed minimal ductule hyperplasia, but single transgenic c-myc mice and transgene-negative littermates were free of pathological abnormalities at 3 months of age (Table 3; Fig 7).

At 7 months of age, histopathology of salivary glands from single transgenic animals revealed minimal serous metaplasia in the sublingual glands of c-myc mice, and mild duct(ule) squamous metaplasia and hyperplasia in the sublingual and submandibular glands of TGF α animals. Wild-type mice at 7 months of age had no apparent abnormalities. No tumors were ever observed in the salivary glands of single transgenic or wild-type mice (up to 10 months for TGF α mice and 15 months for c-myc mice).

To obtain information about the localization of expression of both transgenes within the gland, *in situ* hybridization analysis was performed on sequential sections of salivary glands from TGF α /c-myc animals (Fig. 4, *b*, *d*, and *f*). It revealed a very patchy pattern of expression of the c-myc transgene and scattered expression of the TGF α transgene. Expression was quite different from what we observed in the mammary glands in that only about 5% of salivary gland tissue was positive for both transgenes. Interestingly, areas where expression of both transgenes

was detected appeared hyperplastic and atypical, whereas areas with only one transgene expressed looked quite normal. These areas might represent premalignant areas within the salivary glands, indicating that only when both transgenes are expressed does malignant conversion occur. Immunohistochemical staining of salivary glands from TGF α /c-myc animals was performed with a polyclonal antiserum that recognizes both the endogenous mouse TGF α and the transgene-derived human TGF α . In agreement with *in situ* hybridization data, we observed a scattered distribution of TGF α expression and an association of an intense staining with premalignant hyperplastic atypical nodules (as seen in the sublingual gland in Fig. 5*c*), whereas surrounding areas of normal or hyperplastic salivary gland had little or no TGF α staining. Finally, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) on salivary gland sections revealed a strong staining in areas that coexpressed the transgenes, indicating that DNA synthesis was occurring (Fig. 8).

Discussion

In this study, matings of MT-TGF α and MMTV-c-myc transgenic strains were carried out to investigate the *in vivo*

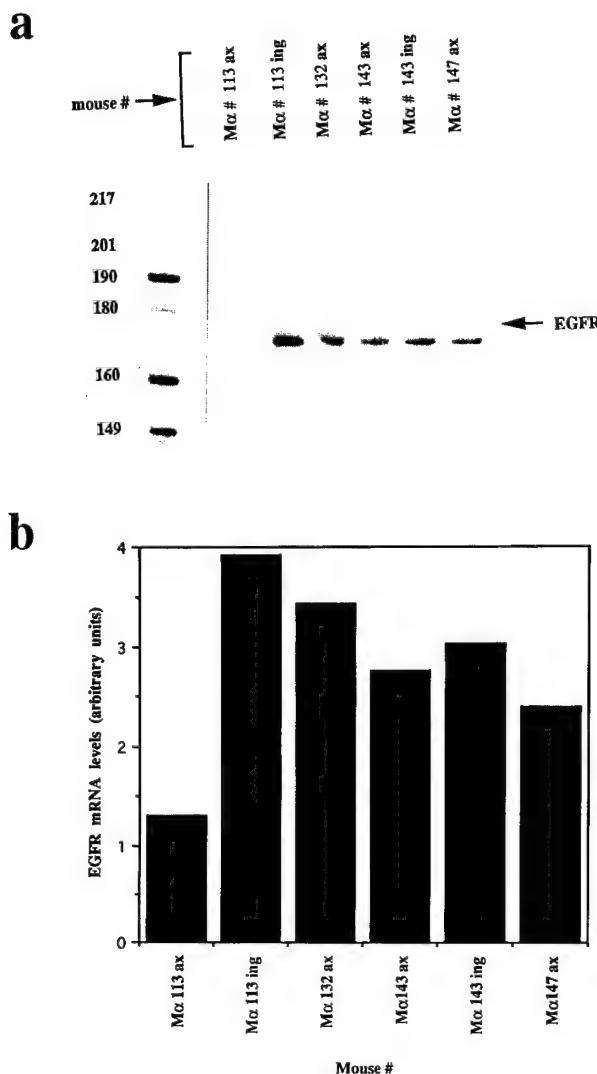


Fig. 6. Expression of the endogenous EGFR mRNA measured by RNase protection assay. RNA levels were compared between mammary gland tumors from double transgenic animals. *b*, a scanned version of the data in *a*. Ma113 through Ma147 denotes the number of each double transgenic mouse used in the assay. *ax*, axillary gland tumor; *ing*, inguinal gland tumor.

interaction of TGF α and c-Myc in mouse mammary gland transformation and confirm our previous *in vitro* studies that showed cooperation between the two. We found that tumorigenesis in mammary glands of double transgenic TGF α /c-myc virgin females and males is strikingly different from single transgenic and wild-type animals and also from multiparous single transgenic TGF α and c-myc mice. In previous studies, both MT-TGF α and MMTV-c-myc single transgenic mice developed polyclonal mammary gland tumors only after a long latency period and multiple pregnancies (21, 24, 35). In contrast, our studies show that both virgin females and males harboring both transgenes develop multiple mammary gland tumors after a mean latency period of only 66 days. All of 20 double transgenic virgin females and males developed rapidly growing mammary gland tumors that could be established in nude mice in the absence of estrogens. Single transgenic virgin TGF α and

Table 3 Summary of histopathological findings in salivary glands of transgenic animals at 3 and 7 months of age

Genotype	3 mos.	7 mos.
TGF α /c-myc	Hyperplasia with atypia, squamous metaplasia and adenoma, and adenocarcinoma	NA ^a
TGF α	Ductule hyperplasia	Ductule hyperplasia and squamous metaplasia
c-myc	Normal	Serous metaplasia
Wild type	Normal	Normal

^a NA, not available (all animals of this genotype are deceased at this time point).

wild-type animals of both sexes did not develop any tumors, whereas about one-half single transgenic c-myc virgin females developed stochastic mammary gland tumors after a long latency period of about 8–12 months. The early onset and multiple tumor formation in double transgenic TGF α /c-myc animals suggests that very few, if any, additional genetic events are necessary for tumorigenesis in our model. In fact, at 3 weeks of age, when the glandular tissue has just started to penetrate the fat pad, the mammary gland is already tumorous.

It is also quite interesting that tumors form in a synchronous manner in our model, so that normal mammary gland tissue is not found at all. Two previous studies have described transgenic models with synchronous tumorigenesis of mammary glands. In the first one, an activated rat *neu* oncogene was expressed from the MMTV-LTR promoter/enhancer, and in the second, the polyoma middle T oncogene was expressed from the same promoter (36, 37). An extremely high level of transgene expression observed in the former strain might have contributed to the phenotype, since MMTV-*neu* transgenic mice made by another group developed only stochastic mammary gland tumors (38). However, the study has been repeated using the same transgene construct with similar results (39). Our model is comparable to the effects of a mutated growth factor receptor, Neu, or the powerful viral protein product of the polyomavirus middle T oncogene that mediates cellular transformation by targeting a number of intracellular signaling pathways (40–43). The fact that overexpression of two normal proteins in the mammary gland of transgenic mice has a similar effect on tumorigenesis in this organ as a mutated, highly active growth factor receptor and a strong viral oncprotein further emphasizes the cooperative effect of TGF α and c-Myc.

An intriguing finding from our studies was that double transgenic TGF α /c-myc males developed mammary cancer in a manner indistinguishable from virgin females. Mammary gland cancer has been described previously in transgenic male mice of MMTV-*neu*, MMTV-v-Ha-ras, MMTV-*int-1*, MMTV-*int-3*, and MMTV-polyomavirus middle T strains. However, tumor onset is typically delayed compared to female mice (35–37, 44, 45). Both TGF α and c-myc are estrogen-inducible genes, and each has been shown to be responsible, at least in part, for estrogen-mediated growth *in vitro* (19, 20). It is, therefore, possible that when both genes are overexpressed *in vivo*, they induce growth of the male mammary gland in the absence of

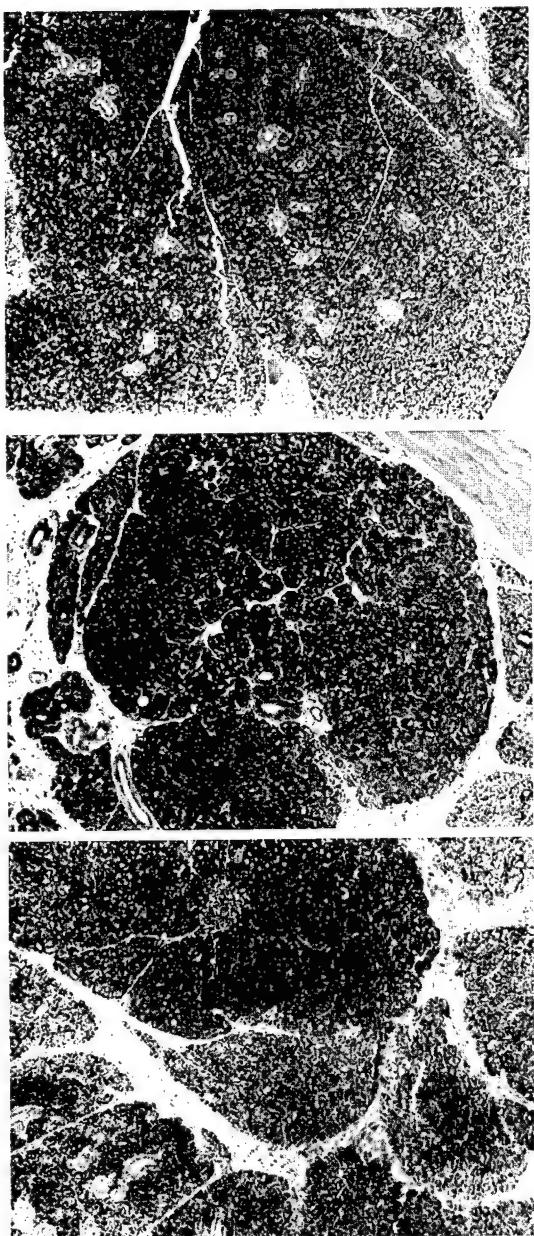


Fig. 7. Hematoxylin & eosin-stained sections of salivary glands. *a*, a normal parotid gland from a wild-type mouse. *b*, a parotid adenoma and *c*, an adenocarcinoma from TGF α /c-myc double transgenic animals. All animals are about 3 months old. $\times 100$.

estrogens. Breast cancer in human males is extremely rare, but in the presence of exogenously applied estrogens, males can develop mammary gland hyperplasias (gynecomastia), thus showing that estrogens can cause proliferation in the male mammary gland in the presence of androgens. It is remarkable that overexpression of two estrogen-induced genes can stimulate the growth and malignant transformation of the male mammary gland as we have seen here. It encourages further studies to dissect the role of, and interaction between, mediators responsible for hormone action on the normal and malignant development of the mammary gland.

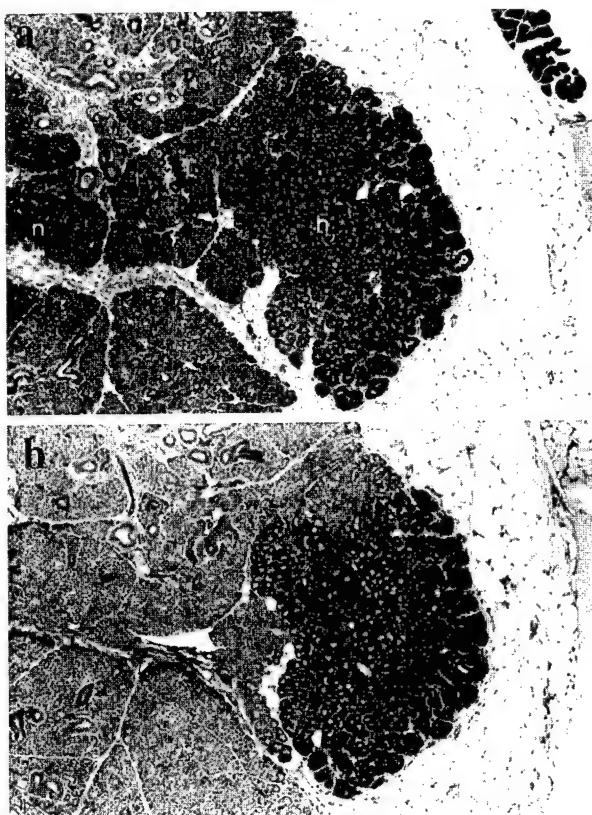


Fig. 8. Immunohistochemical staining of proliferating cell nuclear antigen in salivary glands from a 6-week-old double transgenic TGF α /c-myc animal. *a*, a hematoxylin & eosin-stained section of the salivary gland and *b*, a sequential section stained for proliferating cell nuclear antigen. Note intense proliferating cell nuclear antigen staining in hyperplastic areas of this field. This area in the parotid gland coexpressed the TGF α and c-myc transgenes. *h*, hyperplastic; *n*, normal. $\times 100$.

A cooperative interaction between TGF α and c-Myc also exists in the salivary glands, although malignancies were somewhat less prominent than in mammary glands. In contrast to mammary glands of bitransgenic mice where normal tissue was not found, the salivary glands of double transgenic animals contained normal tissue juxtaposed with hyperplastic areas and frank tumors. However, single transgenic and wild-type animals did not develop salivary gland tumors, whereas they developed mammary gland tumors after a long latency (MMTV-c-myc female mice). Therefore, we conclude that a strong cooperative interaction also exists in the salivary glands.

In situ hybridization analysis revealed that expression of transgenes was more uniform in mammary glands than salivary glands. In fact, only about 5–10% of salivary gland tissue expressed detectable levels of c-Myc (mainly in the parotid gland). Interestingly, areas that expressed both transgenes appeared hyperplastic (salivary glands at 5 weeks) or tumorous (mammary glands at 3 weeks), indicating a requirement for both TGF α and c-Myc in tumorigenesis. In the salivary gland, this was quite clear since areas were found that expressed either TGF α , c-myc, both transgenes, or no transgenes. A progressive tumor onset was associated with a patchy expression of transgenes in the salivary glands. On the other hand, an extremely rapid

tumor onset was associated with a near uniform expression of transgenes in the mammary glands. This might suggest that additional events must occur in the course of the slower tumorigenesis in salivary glands. In the mammary glands, TGF α and c-Myc appear to be sufficient to mediate transformation, although additional events cannot be ruled out. In both glands, there appears to be a selective advantage to express increasing levels of the TGF α transgene in the course of malignant progression. The mechanism of this effect is not known, but an apparently similar phenomenon was observed previously in mouse skin carcinogenesis in transgenic TGF α mice (46).

In summary, TGF α and c-myc are extremely powerful, synergistic-acting genes in breast and salivary gland carcinogenesis in the mouse strains described here. Since TGF α and c-Myc cause uniform transformation of the mammary gland of transgenic mice, this model provides an ideal system to examine possible secondary events for malignant progression/metastasis and characterize the relevance of a deregulated TGF α /EGF receptor pathway in mammary tumorigenesis.

Materials and Methods

Transgenic Mice. The MMTV-c-myc mice used in this study were obtained from Charles River Laboratories (Wilmington, MA); experiments were carried out under a breeding license agreement with Du Pont Medical Products (Wilmington, DE). All mice were rederived and were free of adventitious agents. Line MT100 has a mouse metallothionein I (MT) promoter driving expression of a human TGF α cDNA transgene in an FVB/N inbred genetic background (21). The MMTV-c-myc M line harbors a mouse c-myc gene driven by the mouse mammary tumor virus long terminal repeat promoter/enhancer (MMTV-LTR) in a CD-1 \times C57BL/6J background (24). Both strains have been shown to form stochastic mammary gland tumors after a long latency period and multiple pregnancies. Double transgenic mice were generated by mating the MT-TGF α MT100 line to the MMTV-c-myc M line. Offspring were maintained on 50 mM ZnCl₂ drinking water from the time of weaning (3–4 weeks of age) to induce maximal expression of the TGF α transgene from the metallothionein promoter.

Genotyping of Offspring for Transgenes. DNA was isolated from 1-cm tail biopsies by an overnight proteinase K digestion at 55°C, followed by phenol/chloroform extractions and ethanol precipitation. For Southern analysis, 10 µg of tail DNA was digested overnight at 37°C with the following restriction enzymes: BglII for TGF α ; and BamHI and ClaI for c-myc. After electrophoresis through 0.8% agarose gels and transfer to nitrocellulose, blots were probed with random-primer ³²P-labeled cDNA probes for human TGF α and mouse c-myc. The TGF α probe was a 925-bp EcoRI fragment from the plasmid pTGF α , kindly provided by Dr. Francis Kern (Georgetown University, Washington, DC; Ref. 47). The c-myc probe was a 2400-bp EcoRI-XbaI fragment from the plasmid fpGV-1, generously provided by Dr. MaryLou Cutler (NIH, Bethesda, MD; Ref. 48).

For PCR, 3 µg DNA from tail biopsies was used as a template to amplify transgenes. 3' primers were complementary to sequences in the TGF α and c-myc transgenes, and 5' primers to sequences in the metallothionein (MT) and MMTV promoters, respectively: MT-TGF α 5' primer, 5'-TCG TCC CCG AGC CAG TCG-3'; MT-TGF α 3' primer,

5'-GTC CGT CTC TTT GCA GTT CTT-3'; MMTV-c-myc 5' primer, 5'-CCC AAG GCT TAA GTA AGT TTT TGG-3'; and MMTV-c-myc 3' primer, 5'-GGG CAT AAG CAC AGA TAA AAC ACT-3'. Primers were made by the Lombardi Cancer Center Macromolecular Synthesis and Sequencing Core facility (Georgetown University, Washington DC). PCR was performed using the Perkin Elmer Taq polymerase kit (Perkin Elmer, Norwalk, CT). The TGF α and c-myc transgenes were detected with Southern analysis and/or PCR.

Tumors and Histopathology. Mice were palpated bi-weekly for tumors and sacrificed before tumor sizes reached 10% of body weight. Location and size of each tumor were determined. Tumors were fixed in Bouin's solution for 5–12 h, embedded in paraffin, sectioned at 5 µm, stained with hematoxylin and eosin, and examined to determine histopathological diagnoses. Transplantation of tumors into ovariectomized female NCR nu/nu mice was performed as follows. Tumor-bearing mice were anesthetized with methofane, and tumors were excised aseptically. Tumors were cut into about 1-mm² pieces and inserted s.c. (between nipples nos. 2 and 3) of nude mice under anesthesia.

Whole-Mount Staining. Animals were sacrificed, and the inguinal mammary glands were removed and fixed in 25% glacial acetic acid and 75% ethanol for 60 min at room temperature. After staining overnight in carmine alum solution [1 g carmine and 2.5 g aluminium potassium sulfate (both from Sigma Chemical Co.) in 500 ml water] glands were dehydrated in a series of ethanol washes and finally cleared in toluene. Glands were stored and photographed in methyl salicylate.

Estrogen Receptor Binding Assay. Frozen tumor samples (50–100 mg) were pulverized in liquid nitrogen and homogenized at 0°C in TEDG [10 mM Tris-OH (pH 7.4), 1 mM EDTA, 1 mM DTT, and 10% glycerol] plus 0.5 M NaCl and a cocktail of proteolysis inhibitors (leupeptin at 1 mg/ml, aprotinin at 77 µg/ml, and pepstatin A at 1 µg/ml). Homogenates were centrifuged at 105,000 \times g at 4°C for 30 min to yield a whole-cell lysate, which was then adjusted to 2 mg/ml protein. Lysates were incubated with 10 nM [³H]17 β -estradiol with or without a 100-fold excess of unlabeled estradiol for 16 h at 4°C. Binding was assayed by adding dextran-coated charcoal to adsorb free hormone. After centrifugation, aliquots of supernatant were removed and counted in 10 ml of liquid scintillation fluid in a Beckman liquid scintillation counter. Estrogen receptor-positive control tumors were MCF-7 and MKL-4 breast cancer cell lines grown in nude mice (49). They were generously provided by Dr. Sandy McLeskey (Georgetown University, Washington DC).

RNA Isolation and Analysis. Total RNA was isolated by pulverizing frozen tumors in liquid nitrogen, followed by homogenization in guanidine thiocyanate, acid phenol extraction, and precipitation with isopropanol. TGF α and c-myc transgene expression was assessed by Northern blot hybridization; 15 µg total RNA were electrophoresed through 1.2% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Amersham, Arlington Heights, IL), and probed with a ³²P-labeled random-primer probe. The c-myc probe was generated from the plasmid fpGV-1 (as described above) and the TGF α probe from the plasmid pTGF α -RP as described previously (50).

EGFR expression was determined using RNase protection assays in which ³²P-labeled antisense riboprobes (cRNA) were synthesized *in vitro* from the plasmid pME2.0 for the

EGFR, by linearizing with *Hind*III and transcribing with SP6 polymerase (51). It yields a 170-bp protected EGFR fragment. This plasmid was kindly provided by Dr. M. Rosner (University of Chicago, Chicago, IL). Total RNA (30 µg; EGFR) was hybridized for 12–16 h at 42°C to the ³²P-labeled cRNA probe and treated with RNase A for 30 min at 25°C. The radiolabeled riboprobes protected by total RNA were run on a 6% polyacrylamide/7M urea gel, which was subsequently dried and exposed to autoradiography.

In Situ Hybridization Analysis. To detect localization of transgene expression, *in situ* hybridization analysis was performed on mammary and salivary glands from 3; 6; and 10-week old double transgenic TGF α /c-myc animals. Animals were sacrificed, and glands were fixed in 4% paraformaldehyde in PBS for 24 h. *In situ* hybridization analysis was performed by Molecular Histology, Inc. (Gaithersburg, MD Ref. 52). Probes were generated from plasmids; p.c.-myc20 was generously provided by Dr. S. Thorgeirsson (NIH, Bethesda, MD). For the antisense riboprobe, this plasmid was linearized with EcoRI and transcribed with T7 polymerase. For a sense control riboprobe, the same plasmid was linearized with *Hind*III and transcribed with SP6 polymerase. Plasmid pTGF α -pGem3Z was used to detect the TGF α transgene. For an antisense riboprobe, this plasmid was linearized with *Hind*III and transcribed with T7 polymerase. For a sense control riboprobe, the same plasmid was digested with EcoRI and transcribed with SP6 polymerase.

Immunostaining. Tissues were fixed in Bouin's solution for 5–12 h, embedded in paraffin, and sectioned. After treatment with 0.02% trypsin for 15 min, sections were incubated overnight at room temperature with a 1:20,000 dilution of a rabbit polyclonal antiserum generated against a rat pro-TGF α intracellular peptide (residues 137–159). The antibody was kindly provided by Dr. Larry Gentry (Medical College of Ohio, Toledo, OH). TGF α was localized using the Vectastain Rabbit Elite kit (Vector Laboratories, Burlingame, CA), as described previously (50). PCNA immunostaining was performed as described previously (53).

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apoptosis have been identified (Askew *et al.*, 1991; Harrington *et al.*, 1994). In mouse fibroblasts for example, apoptosis induced by c-Myc was inhibited by various growth factors, such as insulin-like growth factors and PDGF, whereas EGF and bFGF were ineffective. In the current study, we show that TGF α can function as a survival factor for mammary epithelial cells which overexpress c-Myc. We observed that apoptosis occurred in mouse mammary gland tumors and in their derived epithelial cell lines only in cases where c-Myc was overexpressed in the absence of TGF α overexpression. Furthermore, when c-Myc overexpressing cells were treated with TGF α *in vitro*, apoptosis was greatly decreased. Our results suggest an explanation for the cooperative interaction between TGF α and c-Myc in tumorigenesis: both factors stimulate anchorage dependent proliferation and anchorage independent growth, and in addition, TGF α suppresses c-Myc-induced apoptosis.

Results

Programmed cell death occurs only in mammary gland tumors from c-myc single transgenic mice

To examine whether apoptosis was a factor in the cooperation between TGF α and c-Myc in tumorigenesis, we measured apoptosis in five mammary gland tumors from each of the three transgenic mouse strains (double transgenic TGF α /c-myc mice, and single transgenic TGF α and c-myc mice). *In situ* nick end-labeling of nucleosomal fragments by Klenow DNA polymerase I revealed that apoptosis was occurring in mammary gland tumors from c-myc transgenic animals and not in mammary tumors from single transgenic TGF α animals or double transgenic TGF α /c-myc animals. As shown in Figure 1c, only tumors from c-myc mice exhibited scattered cells with positive staining. When apoptosis was quantitated by counting apoptotic cells in 20 random fields of each tumor type (400 \times magnification) we observed that tumors from c-myc mice had 23.0 \pm 2.8 apoptotic cells per field whereas tumors from TGF α and TGF α /c-myc animals had 0.3 \pm 0.2 and 1.2 \pm 0.5 labeled cells per field respectively.

Generation of cell lines from mammary gland tumors

We have generated three cell lines from mammary gland tumors arising in double and single transgenic mice. Our intent was to use them to verify our findings in mammary gland tumors *in vivo* and examine further the molecular mechanisms underlying the cooperation between TGF α and c-Myc. The following nonclonal cell lines were generated: TGF α /Myc#75 was derived from a tumor arising in a double transgenic virgin female (TGF α /c-myc animal number 75), Myc#83 was derived from a c-myc virgin single transgenic female (c-myc animal number 83) and TGF α #13 from a multiparous TGF α single transgenic female (TGF α animal number 13). Additional cell lines from tumors arising in c-myc and TGF α /c-myc animals have recently been isolated and were used where noted to confirm our findings with the first three cell lines.

Expression of cytokeratins and morphology of cell

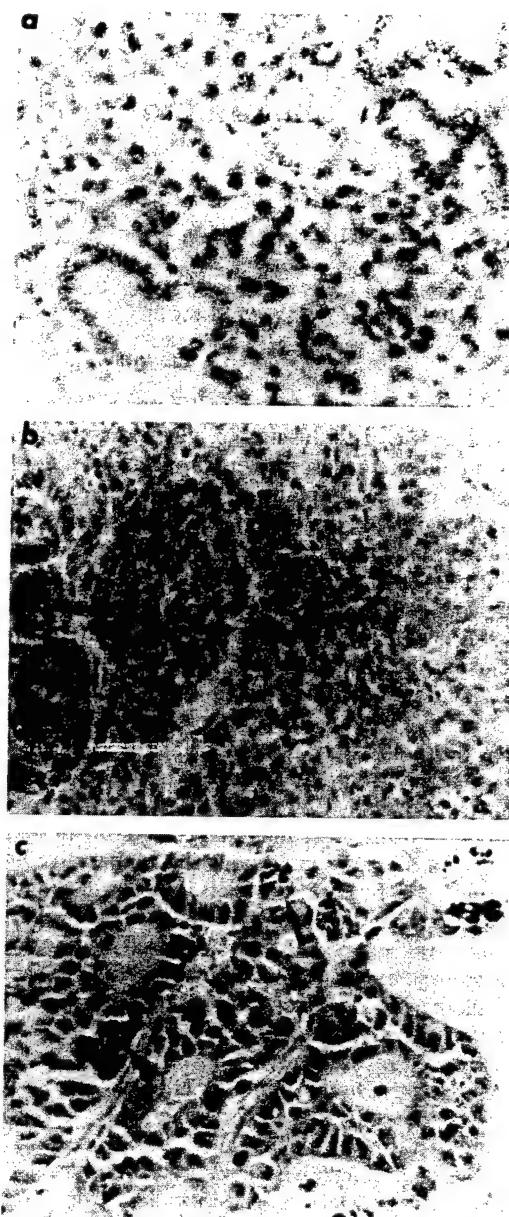


Figure 1 Detection of apoptosis in mammary gland tumors from transgenic mice. Tumor sections were analysed by *in situ* nick end-labeling of DNA fragments. (a) shows a tumor from a double transgenic virgin TGF α /c-myc mouse, (b) a tumor from a single transgenic multiparous TGF α mouse and (c) a tumor from a single transgenic c-myc mouse. Note cytoplasmic staining in scattered cells of the tumor from a c-myc single transgenic animal (arrows, c) indicating DNA fragmentation

lines grown as subcutaneous tumors in nude mice was used to verify epithelial origins of the cell lines. The single transgenic cell lines TGF α #13 and Myc#83 were positive for keratin 14 at the mRNA level (not shown). TGF α /Myc#75 cells apparently did not express keratin 14, but positive immunofluorescent signal was observed in these cells with a pan-keratin antibody (not shown). All three lines also gave rise to tumors in nude mice that had a very distinct epithelial morphology. We therefore conclude that all three lines are epithelial, but that line TGF α /Myc#75 has probably lost expression of some of its keratins. This is not without precedent, since human breast

carcinoma cell lines, especially hormone independent lines, have been described to do the same (Sommers *et al.*, 1989, 1992).

Expression of the TGF α and c-myc transgenes was also assessed by Northern analysis (not shown). The single transgenic lines, TGF α #13 and Myc#83, expressed only the TGF α or c-myc transgenes, respectively. In contrast, the double transgenic cell line, TGF α /Myc#75, expressed both transgenes. Therefore, all three cell lines expressed the expected transgenes at the mRNA level. In addition, expression of the TGF α transgene was upregulated by ZnC12 and CdC12 as expected, since the TGF α transgene is expressed from the heavy metal-inducible metallothionein promoter (Jhappan *et al.*, 1990). The endogenous c-myc gene was downregulated in the two cell lines expressing the c-myc transgene (TGF α /Myc#75 and Myc#83), consistent with a negative autoregulation of the c-Myc protein on its own promoter, as has been described previously (Penn *et al.*, 1990).

In order to assess ploidy of the cell lines, cells were stained with propidium iodide and analysed by FACS. Each cell line was tested at two or three different timepoints (between passages 6 and 31). Only the double transgenic line TGF α /Myc#75 was found to be aneuploid. It was tetraploid at all three timepoints tested. In contrast, the two single transgenic lines were diploid at the time points tested (not shown).

Apoptosis occurs in c-Myc overexpressing mammary tumor cells in vitro

Tumor derived cell lines were tested for their ability to undergo apoptosis by two independent methods. For the first method, cytoplasmic DNA fragments were isolated and run on agarose gels. In this assay, the Myc#83 line was positive with a characteristic nucleosomal ladder whereas both TGF α #13 and TGF α /Myc#75 were negative (Figure 2a). Those results were confirmed by an ELISA apoptosis assay that is based on detecting histone-associated DNA in cytoplasmic cell lysates via a peroxidase catalyzed color change (A_{405}). CEM cells (a T cell leukemia cell line) treated with 10^{-7} M dexamethasone served as a positive control (Catchpoole and Stewart, 1993; not shown). The Myc#83 line showed a high degree of apoptosis, whereas the other two had levels close to background (Figure 2b). Five additional cell lines derived from Myc-single transgenic tumors also showed a propensity to undergo apoptosis as determined by the apoptosis ELISA (not shown). The appearance of apoptotic Myc#83 cells under conditions of EGF deprivation or TGF β 1 treatment was further confirmed by observing morphological changes which are characteristic of apoptosis (Figure 2c). The cells displayed prominent apoptotic bodies with concomitant reduction of cytoplasm and altered nuclear morphology.

When the Myc#83 line was treated with the growth factors TGF α , EGF, IGF-I or bFGF, apoptosis was inhibited up to 75% as measured by the ELISA assay (Figure 3a). In contrast, treatment with TGF β 1 resulted in elevated levels of apoptotic DNA, even in the presence of EGF. The effects of both TGF α and TGF β 1 on apoptosis were concentration dependent (Figure 3b and c), with maximal responses at 10 ng/ml and 100 pM, respectively.

Additional evidence for the importance of the TGF α /EGF receptor system was provided by using a synthetic inhibitor of EGF receptor tyrosine kinase activity (PD153035). PD153035 has been shown to

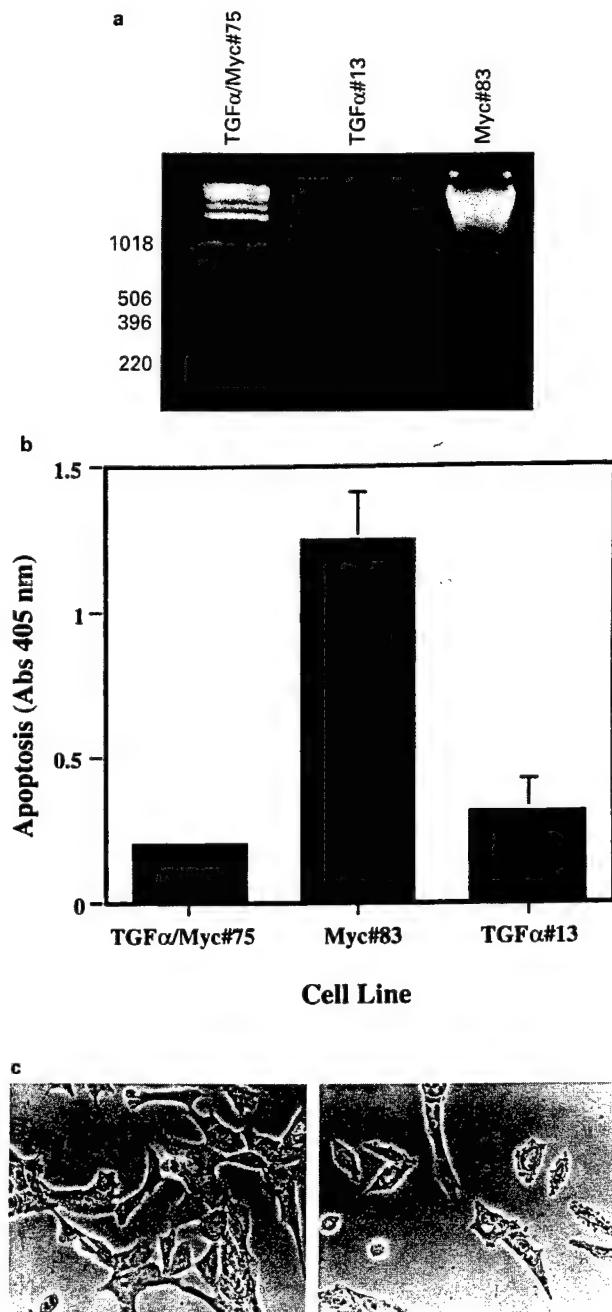


Figure 2 Detection of apoptosis in tumor-derived cell lines. (a): Nucleosomal ladder. DNA was isolated from cytoplasmic fractions of transgenic tumor cell lines and run on a 1.8% agarose gel which was stained with ethidium bromide. Note characteristic nucleosomal DNA with a size interval of approximately 180 bp. (b): ELISA-apoptosis assay. Apoptotic DNA fragments were detected in cytoplasmic lysates via a histone-DNA ELISA. Peroxidase substrate conversion was quantitated by measuring absorbance at 405 nm. (c): Morphological changes in apoptotic Myc#83 cells. Cells on the left side were grown in media containing EGF. The right side is representative of cells which have become apoptotic via EGF deprivation or TGF β 1 treatment, for 24 h. Note the characteristic reduction in cell size due to cytoplasmic blebbing (apoptotic bodies).

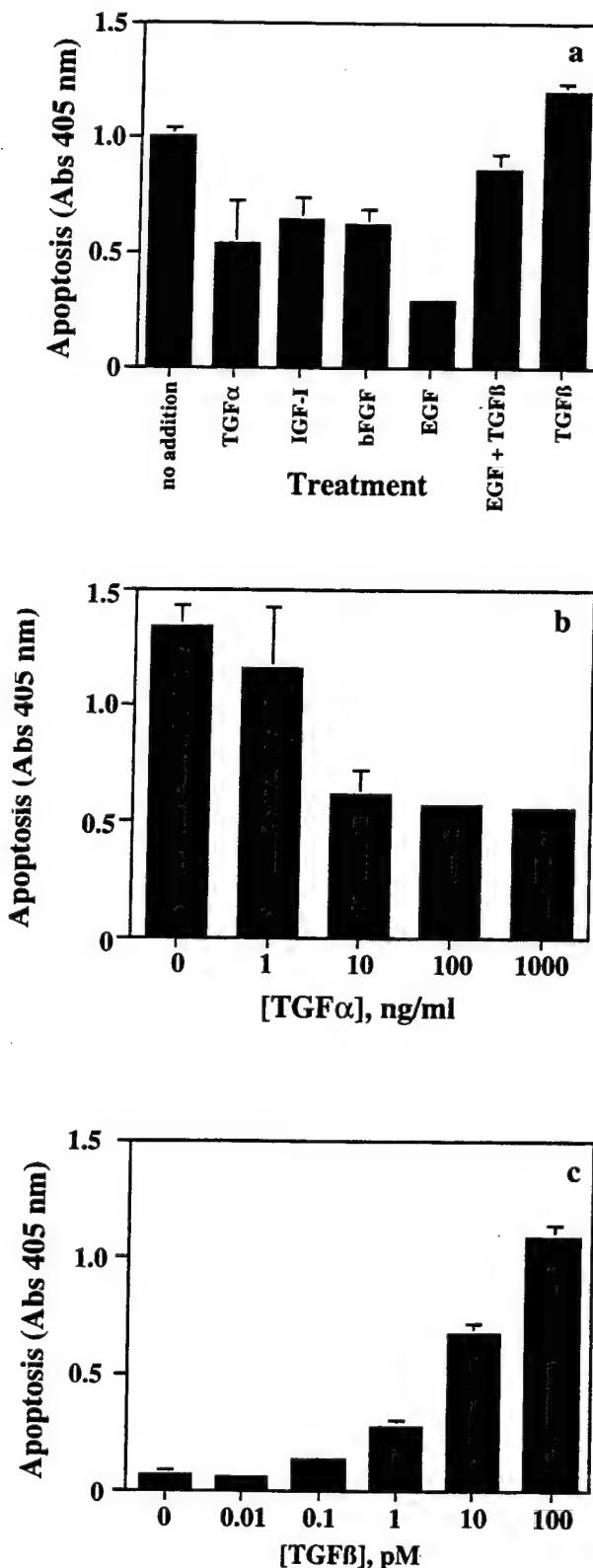


Figure 3 Effects of growth factors on apoptosis in the Myc#83 line. (a) shows the effects of TGF α (5 ng/ml), IGF-I (50 nM), bFGF (8 ng/ml), EGF (10 ng/ml), TGF β (100 pM) or EGF + TGF β on apoptosis. (b) shows that the inhibitory effect of TGF α on apoptosis in the Myc#83 cell line is concentration dependent. (c) demonstrates that the stimulatory effect of TGF β on apoptosis is also concentration dependent. In all three panels, cells were treated for 24 h prior to harvest for apoptosis ELISA. Each point represents the mean (\pm SE) of two determinations.

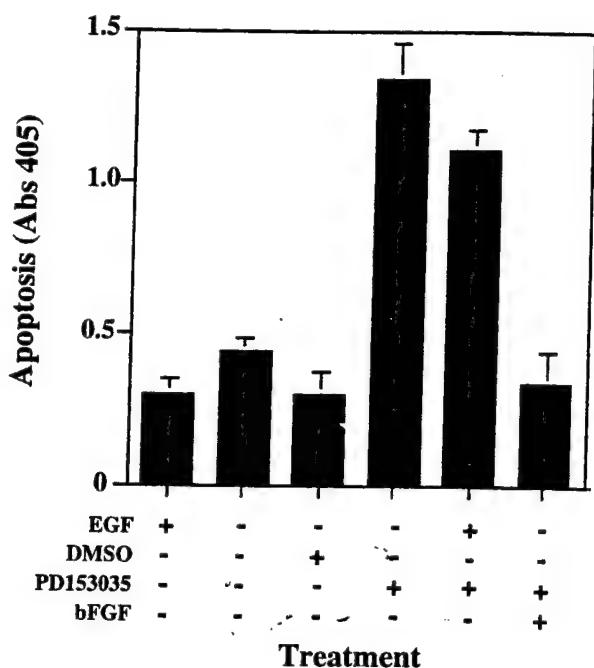


Figure 4 Induction of apoptosis in the TGF α /Myc#75 line by PD153035, a specific inhibitor of EGF receptor tyrosine kinase activity. Cells were incubated for 24 h with the indicated additions (10 ng/ml EGF or bFGF, 10 μ M PD153035, 1 μ l/ml DMSO as a control) and apoptosis was measured via ELISA assay. $n=4$ (\pm SE)

specifically downregulate the tyrosine phosphorylation status of the EGFR (Fry *et al.*, 1994). We verified this in our cell system by an anti-phosphotyrosine Western blot which showed that a 170 kb species was reduced greater than 90% in cells treated with the compound, while other phosphotyrosine bands remained constant (not shown). The TGF α /Myc#75 cells became apoptotic when exposed to PD153035 for 24 h (Figure 4). Removing EGF from the growth media of these cells did not affect viability, but exposure to PD153035 in either the presence or absence of EGF induced apoptosis. In contrast, bFGF, which acts through a different receptor tyrosine kinase, could rescue the cells from the effects of the drug.

Anchorage dependent (ADG) and anchorage independent (AIG) growth analysis of tumor derived cell lines

The TGF α /Myc#75 double transgenic cell line had the fastest ADG growth rate *in vitro* under normal growth conditions (doubling time of $16.7 \text{ h} \pm 0.4 \text{ h}$). Myc#83 and TGF α #13 had much longer doubling times of $33.4 \text{ h} (\pm 1.7)$ and $35.0 \text{ h} (\pm 0.82)$ respectively when grown in normal media containing EGF (Figure 6a). Growth rates of all three cell lines were similar when EGF was replaced with TGF α (not shown). The two cell lines that overexpress TGF α (TGF α /Myc#75 and TGF α #13) were able to grow in the absence of exogenous EGF, but with a significantly reduced growth rate (Figure 5a). Two additional TGF α /Myc cell lines also exhibited relatively fast growth rates and were not dependent on exogenous EGF for growth or survival, similar to TGF α /Myc#75 (not shown). In

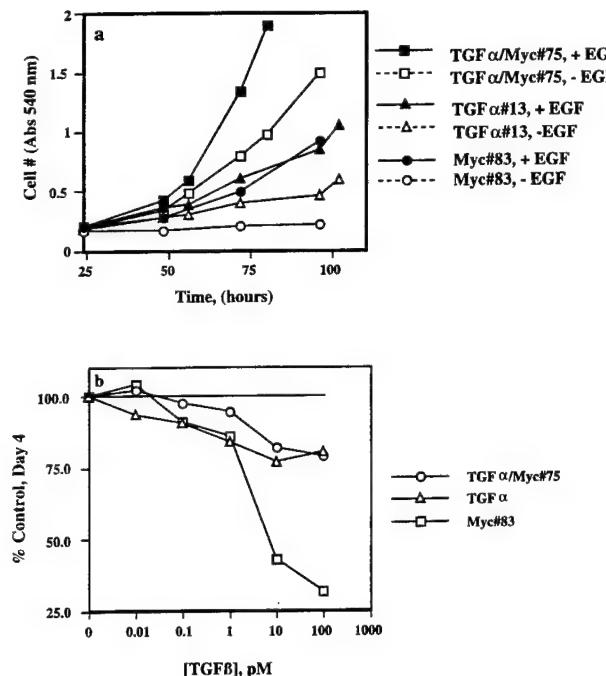


Figure 5 Co-expression of TGF α and Myc results in cooperative growth stimulus under anchorage dependent conditions. (a): Cells were grown in the presence or absence of EGF in 96 well plates for the indicated times and stained with crystal violet. (b): Cells were grown in the presence of EGF and increasing concentrations of TGF β 1 for 3 (TGF α /Myc#75) or 4 (Myc#83, TGF α #13) days and then stained with crystal violet. For (a) and (b), $n=8$ (\pm SE)

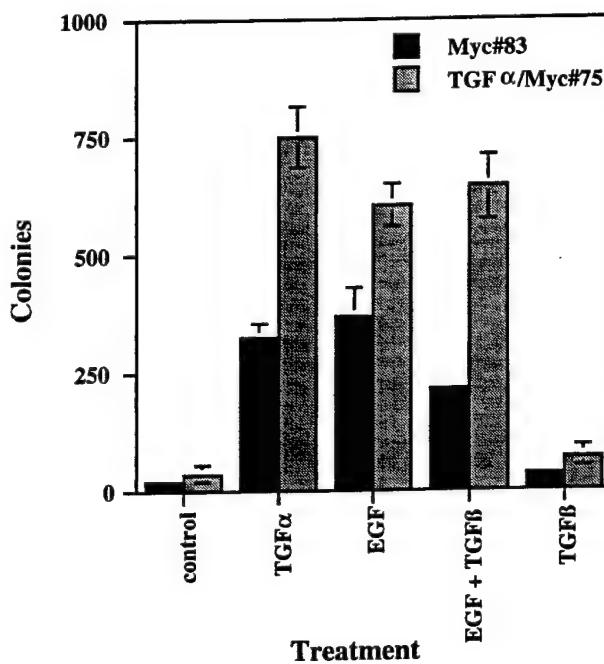


Figure 6 Co-expression of TGF α and Myc results in cooperative growth stimulus under anchorage independent conditions. Cells were suspended in 0.3% agar with 10% FCS and the following additions: TGF α (5 ng/ml), EGF (10 ng/ml), TGF β 1 (100 pM) or EGF + TGF β 1. Colonies were counted on day 7 (TGF α /Myc#75) or day 10 (Myc#83). $n=3$ (\pm SD). Results for TGF α #13 are not shown since that cell line did not grow well in soft agar under any of the above conditions

contrast, Myc#83 cells were completely dependent on exogenous EGF and showed no significant growth in its absence. However, FACS analysis demonstrated that Myc#83 cells were not arrested in G0/G1 when deprived of EGF (not shown). Those results are consistent with the hypothesis that Myc overexpressing cells are unable to withdraw from the cell cycle and undergo apoptosis in the absence of EGF.

All three cell lines exhibited concentration dependent inhibition by TGF β 1 under ADG conditions, but sensitivity to the growth factor varied (Figure 5b). At the highest concentrations (10–100 pM), TGF α /Myc#75 and TGF α #13 were marginally responsive to TGF β , with about 20% fewer cells in treated wells than in untreated controls after 3–4 days in culture. In contrast, Myc#83 cells were quite sensitive to high concentrations of TGF β 1, reflecting the observation that TGF β induces apoptosis in these cells (Figure 3a,c).

TGF α /Myc#75 and Myc#83 cells both grew well under AIG conditions in the presence of exogenous EGF or TGF α (Figure 6), whereas TGF α #13 cells grew poorly in soft agar. The effects of those growth factors on Myc#83 cells are similar to published results for other Myc-overexpressing breast cells (Telang *et al.*, 1990; Valverius *et al.*, 1990). The rate of colony formation and growth was much higher for the TGF α /Myc#75 cells, and a dose response curve showed that those cells were extremely sensitive to TGF α , with optimal induction by only 0.1 ng/ml of the growth factor. Maximal colony formation by the Myc#83 line occurred with 10–30 ng/ml TGF α (not shown). Addition of TGF β 1 significantly reduced the number of Myc#83 colonies stimulated by addition of EGF, but had no effect on TGF α /Myc#75 colony formation (Figure 6).

Tumorigenicity of tumor derived cell lines

Cells were injected into female *nu/nu* mice to establish their *in vivo* tumorigenicity and growth rate. All three cell lines grew readily in nude mice, but with different latency times. The double transgenic line TGF α /Myc#75 formed tumors with a latency period of only 4 weeks, while the single transgenic TGF α #13 and Myc#83 lines formed tumors with a latency period of about 9 weeks. None of the cell lines appeared to have metastatic capabilities over the period of time the tumors were allowed to grow (About 2 months for TGF α /Myc#75 and 3 months for TGF α #13 and Myc#83).

Discussion

Myc overexpression (achieved by gene amplification, translocations and other means) has been strongly implicated in the genesis of many types of human tumors including breast cancer (Bonilla *et al.*, 1988; Cole, 1986; Escot *et al.*, 1986; Garcia *et al.*, 1989; Mariani-Constantini *et al.*, 1988). However, since deregulated c-Myc expression can promote cell death via apoptosis, it is likely that the apoptotic pathway(s) induced by c-Myc must be inhibited or inactivated to achieve aggressive tumor formation. That may be accomplished, directly or indirectly, by secondary

events which alter the cell environment (such as growth factor secretion) or gene expression (such as mutations in downstream effectors). In accordance with that hypothesis, we detected apoptosis only in mammary gland tumors that expressed the c-myc transgene alone without the TGF α transgene. Tumors from TGF α single transgenic or TGF α /c-myc double transgenic mice did not contain apoptotic cells. Van Dyke and co-workers have proposed a similar 'multi-hit' hypothesis of tumor formation based on studies of SV40 T antigen-induced brain tumors (Symonds et al., 1994). In that system they found that wild type T antigen induced rapidly growing, aggressive tumors, whereas a mutated form of the protein which only interfered with pRb function produced very slow growing tumors which displayed a high percentage of apoptotic cells. In contrast, expression of the mutant T antigen in a p53-null background resulted in tumors which were indistinguishable from those induced by the wild type protein. Taken together, the results suggest that the first event in cancer initiation stimulates both proliferation and apoptosis and that a secondary event which blocks apoptosis is necessary for aggressive tumor formation.

Cell lines derived from the tumors provided an *in vitro* confirmation of our *in vivo* observations. Apoptosis was observed only in the cell line derived from a Myc single transgenic animal (Myc#83), while the two cell lines overexpressing TGF α (TGF α /Myc#75 and TGF α #13) did not undergo apoptosis under normal culture conditions. TGF α /Myc#75 cells only became apoptotic when exposed to a specific inhibitor of EGF receptor tyrosine kinase activity (PD153035), suggesting that these cells were dependent on autocrine stimulation by TGF α for survival. Analogously, exogenous TGF α inhibited apoptosis in the Myc#83 cells. Results from both mammary gland tumors and their derived cell lines are therefore in good agreement and mirror previous studies which have shown that apoptosis was induced when c-Myc was overexpressed (Askew et al., 1991; Evan et al., 1992).

Our findings could provide at least a partial explanation for why TGF α and c-Myc cooperate in mammary gland tumorigenesis in the powerful way we described previously (Amundadottir et al., 1995). In that study, single transgenic virgin c-Myc mice developed mammary gland tumors at around 10 months of age and virgin single transgenic TGF α animals never developed mammary gland tumors. In contrast, double transgenic TGF α /c-myc mice exhibited a tumor latency that was shortened to only 66 days, and mammary gland tissue from mice as young as 3 weeks grew readily as a tumor in nude mice. A similar synergism was observed in a WAP-TGF α × WAP-Myc double transgenic model (Sandgren et al., 1995). In that report, as well as a study involving the MT100TGF α strain used in our model (Smith et al., 1995), it was also observed that TGF α overexpression inhibited post-lactational involution, a process dependent on apoptosis. Those observations lend further credence to the hypothesis that TGF α can act as a survival factor in the mammary gland.

TGF α has not been shown previously to inhibit c-Myc-mediated apoptosis but insulin like growth factors (IGF-I and IGF-II) and platelet derived growth factor (PDGF) acted as survival factors for Rat-1 fibroblasts which overexpressed c-Myc (Harrington et al., 1994).

Interestingly, EGF could not inhibit c-Myc-induced apoptosis in those cells, even though they expressed functional EGF receptor. Although the reasons for this discrepancy are not known, it is most likely the result of cell type specificity. That assumption is supported by the observation that EGF could act as a survival factor for nontransformed mammary epithelial cells which were serum starved or grown to confluence (Merlo et al., 1995). Our data from the Myc#83 tumor cell line indicate that EGF, bFGF and IGF-I can also inhibit c-Myc-mediated apoptosis, suggesting that they could potentially cooperate with c-Myc in mammary tumorigenesis as well.

Mutations in the p53 gene may also cooperate with c-Myc in tumorigenesis, since p53 has been shown to be required for Myc-mediated apoptosis in some, but not all cases (Sakamuro et al., 1995; Hsu et al., 1995; Hermeking and Eick, 1994). In addition, upregulation of *bcl-2* gene expression (a death suppressor), downregulation of *bax* gene expression (a death promotor) or abrogated expression of other proteins involved in Myc-induced apoptosis might be involved. *Bcl-2* has been shown to cooperate with c-Myc in transformation *in vitro* and *in vivo* (Bissonette et al., 1992; Fanidi et al., 1992; Strasser et al., 1990; Wagner et al., 1993), and *Bax* gene expression may be regulated by c-Myc, since its promotor contains several putative Myc binding sites (Miyashita and Reed, 1995). However it is not known whether TGF α can directly influence the apoptotic machinery of the cells. The effects of TGF α and TGF β on expression of p53, Bcl-2, Bax and other proteins involved in apoptosis are currently being investigated.

A cooperative growth stimulus also appears to contribute to the synergism between TGF α and c-Myc in mammary tumorigenesis. The doubling time of TGF α /Myc#75 cells was approximately half that of cells expressing only one of the transgenes, and their growth in soft agar and nude mice was much more aggressive than the other two cell lines. Taken together, the data suggest that one aspect of the positive interaction between TGF α and c-Myc in tumorigenesis might be via upregulation of genes that control progression through the cell cycle. Collectively, these gene products might account for high growth rates and malignant progression. Potentially, coexpression of TGF α and c-Myc could also alleviate negative control on growth and transformation.

In vitro studies with the tumor-derived cell lines suggest that may be the case. TGF β inhibits the growth of most epithelial cells, including mammary epithelial cells (Daniel et al., 1989; Jhappan et al., 1993; Pierce et al., 1993; Silberstein and Daniel, 1987; Valverius et al., 1989; Zugmaier et al., 1989). However, the TGF α /Myc#75 line was only marginally responsive to TGF β in ADG assays and was insensitive to the growth factor under anchorage independent conditions. In contrast, Myc#83 cells grown on plastic were quite sensitive to TGF β , and their rate of colony formation in soft agar was significantly reduced in the presence of TGF β . Apoptosis assays revealed that Myc#83 cells were not merely growth-inhibited by TGF β , but rather they were stimulated to undergo apoptosis, even in the presence of a survival factor (EGF). Induction of apoptosis by TGF β has previously been reported for

some other cell types, including normal and malignant ovarian epithelial cells (Havrilesky *et al.*, 1995), endometrial cells (Rotello *et al.*, 1991), rat prostate cells (Martikainen *et al.*, 1990), normal and transformed hepatocytes (Oberhammer *et al.*, 1992), and leukemia cells (Selvakumaran *et al.*, 1994a,b; Taetle *et al.*, 1993). Furthermore, mammary glands from pregnant WAP-TGF β transgenic mice showed high levels of apoptosis with a subsequent lack of secretory lobule development (Korden *et al.*, 1995). Since TGF β expression is elevated in human tumor cells compared to normal mammary tissue and protein levels are positively correlated with disease progression (Gorsch *et al.*, 1992), breast tumor cells must develop the ability to grow in the presence of relatively high concentrations of TGF β . The results from our *in vitro* studies indicate that cells which overexpress only c-Myc would not have that ability.

Our results suggest a new role for TGF α as a survival factor in breast cancer. We therefore conclude that the strong synergism of TGF α and Myc in mammary gland tumorigenesis is in fact due not only to a dual growth stimulus, but to the ability of TGF α to suppress a negative aspect of Myc overexpression.

Materials and methods

Transgenic animals

Transgenic mice were generated as described previously by mating the MT100 TGF α strain to the MMTV-c-myc M strain (Amundadottir *et al.*, 1995). The four resulting genotypes were: TGF α /c-myc double transgenic mice, TGF α single transgenic mice, c-myc single transgenic mice, and wild type mice. Tumors were observed to form in each strain as follows: in TGF α /c-myc virgin females and males with a latency of about 66 days; in multiparous single transgenic TGF α females with a latency of about 10 months; and in virgin or multiparous single transgenic c-myc females with a latency of about 10 months.

Detection of apoptosis in tumors

The occurrence of apoptosis in mammary gland tumors was detected by *in situ* nick end-labeling of nucleosomal DNA fragments (Ansari *et al.*, 1993). Paraffin embedded tumor sections were deparafinized in a series of xylene and ethanol washes. This was followed by a 0.3% H₂O₂ treatment for 30 min to inactivate endogenous peroxidases, after which slides were immersed in buffer A for 5 min (50 mM Tris pH 7.5, 5 mM MgCl₂, 0.76 mM 2-mercaptoethanol and 0.005% BSA). Subsequently, slides were incubated with Klenow enzyme (50 U/ml, Boehringer Mannheim, Indianapolis, IN), 5 μ M biotinylated dUTP (Boehringer Mannheim) and 2 μ M dATP, dGTP and dCTP (Promega, Madison, WI) in buffer A for 60 min at 37°C. After washing slides in PBS, they were incubated with solution AB (ABC kit, Biomeda, Foster City, CA), rewashed in PBS and stained with diamino benzidine (DAB, Sigma, St. Louis, MO). Finally, the slides were counterstained with aqueous methyl green (Sigma), dehydrated and mounted.

Primary cultures from tumors

Tumor bearing transgenic animals were sacrificed and tumors were excised aseptically. Tumors were then cut into about 1 mm³ pieces and digested overnight at 37°C in

DMEM-F12 (Biofluids, Rockville, MD) with 10% fetal calf serum (FCS, Biofluids), 5 ng/ml EGF (Upstate Biotechnology Incorporated[UBI], Lake Placid NY), 10 μ g/ml insulin (Biofluids) supplemented with 1 mg/ml collagenase type 1A (Sigma), antibiotics and fungizone (Biofluids). The following day cells were pelleted by centrifugation and washed three times in growth media (DMEM-F12 with 2.5% FCS, 5 ng/ml EGF, 10 μ g/ml insulin and antibiotics). Cells were plated at 1–2 \times 10⁶ cells per T75 flask in growth medium. Fungizone was used in the cell medium for the first 2–3 weeks to prevent fungal contamination. Media were changed every 2–3 days and fibroblast overgrowth was prevented by differential trypsinization of cultures until fibroblasts were no longer observed (based on morphology). When epithelial cells were about 60–70% confluent (after 2–3 months of growth), the cultures were passed at 1:2 dilutions with dispase (Boehringer Mannheim). At later passages cells were split with trypsin (Gibco BRL, Gaithersburg, MD) twice a week at 1:5 to 1:50, depending on the line.

RNA isolation and analysis

Cultured cells were harvested by rocking plates with guanidine thiocyanate for 5–10 min. RNA was extracted with acid phenol and precipitated with isopropanol. Ten μ g total RNA were electrophoresed through 1.2% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Amersham, Arlington Heights, IL) and probed with ³²P-labeled riboprobes generated with the Riboprobe II Core System (Promega) from the following plasmids: pTGF α -pGEM3Z linearized with HindIII and transcribed with T7; c-myc-pGEM4Z linearized with EcoRI and transcribed with T7; and pmK14-pGEM3 (mouse keratin#14) linearized with HindIII and transcribed with SP6 polymerase. Labeled pBluescript polylinker was hybridized with the 28S RNA as an internal loading control for Northern analysis (Witkiewicz *et al.*, 1993).

Growth assays

Anchorage dependent growth assays were performed in 96-well plates (Costar, Cambridge, MA). Cells were plated at a density of 1500 cells per well and were cultured in normal growth media, with or without EGF (10 ng/ml). At various time points (two per day for 4 days), the plates were stained with crystal violet (Sigma, 0.5% in 30% MeOH), rinsed with water and dried. At the end of the experiment, the dye was redissolved in 0.1 M sodium citrate in 50% ETOH and the absorbance at 540 nm was measured with an MR700 plate reader (Dynatech Laboratories Inc.). Doubling times were calculated from the slope of the line generated by plotting log (absorbance) vs time. In order to test the sensitivity of the cells to TGF β 1, cells were also plated in normal growth media with EGF plus TGF β 1 (0.01–100 pm [R&D Systems, Minneapolis, MN]). When TGF β was used as a treatment, the media were changed every other day and cell number was measured on day 3 (TGF α /Myc#75) or 4 (TGF α #13 and Myc#83).

The cell lines were also tested for their ability to grow under anchorage independent conditions. Cells (10⁴) were suspended in 0.3% Bactoagar (Difco, Detroit MI) and seeded into 35 mm dishes over a 0.8% agar base layer in IMEM plus 10% FCS with the following additions: TGF α (10 ng/ml, UBI), EGF (10 ng/ml), TGF β 1 (100 pm), or TGF β 1 and EGF together. Every other day, 300 μ l of media with growth factors was added to each plate. After 7–10 days, colonies larger than 40 μ m in diameter were counted with an Omnicron 3600 image analysis system (Artek Systems Corp., Farmingdale NY).

Tumorigenicity of cell lines

Cell lines were injected into female NCR *nu/nu* mice in order to determine whether they retained tumorigenic potential. About 10^6 cells were injected subcutaneously (between nipples number 2 and 3, and 4 and 5) into nude mice under anesthesia. Two to four sites were injected per animal.

Detection of apoptosis in cell lines

Apoptosis in the cell lines was detected by an apoptotic cell death ELISA assay (Boehringer Mannheim) and by visualization of nucleosomal laddering in cytoplasmic fractions of cells (Kamesaki *et al.*, 1993). The ELISA detects cytoplasmic nucleosomal DNA fragments with antibodies directed against histones and DNA. Cells were plated in 6-well plates (1.7×10^5 cells/well) and treated 24 h later. Treatments consisted of normal growth media without EGF plus the following additions: TGF α (1–1000 ng/ml), bFGF (10 ng/ml, UBI), IGF-I (50 mM, UBI), EGF (10 ng/ml), or EGF plus TGF β (10 ng/ml and 0.01–100 pM, respectively). The TGF α /Myc#75 cells were also treated with PD 153035 (10 μ M, Park Davis), a specific inhibitor of EGF receptor tyrosine kinase activity (Fry *et al.*, 1994). Treatment with DMSO (μ l/ml) served as a negative control since the stock drug was suspended in DMSO. Twenty-four hours later, cytoplasmic lysates were prepared from the cells. The ELISA plate was coated overnight (4°C) with the first antibody (anti-histone) and then incubated with lysis buffer for 30 min at room temperature (RT). The wells were washed three times and then incubated with 100 μ l cytoplasmic lysate for 90 min (RT). Wells were washed again and incubated with the peroxidase-linked second antibody (anti-DNA). Following the final wash, ABTS peroxidase substrate was added and color development was detected by measuring absorbance at 410 nm. CEM cells (T cell leukemia cell line) treated with dexamethasone (Catchpoole and Stewart, 1993) served as a positive control.

Internucleosomal cleavage of the DNA is a hallmark of apoptosis and can be observed as a 'ladder' in agarose gels. DNA was isolated from cytoplasmic fractions of the cells and was run out on 1.8% agarose gel which was stained with ethidium bromide to visualize the DNA ladder (Kamesaki *et al.*, 1993). Cells that are undergoing apoptosis show a

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characteristic DNA 'ladder' in this assay whereas other cells do not contain DNA in their cytoplasm and are therefore negative.

FACS analysis

Cell nuclei were analysed by the detergent-trypsin method (Vindelov *et al.*, 1983) with a Fluorescent Activated Cell Sorter (FACS) to obtain cell cycle histograms and to determine ploidy. Approximately 10^6 cells were pelleted and resuspended in 100 μ l of citrate buffer (250 mM sucrose, 40 mM trisodium citrate, 0.05% v/v DMSO, pH 7.6) and stored at –70°C before analysis. For cell cycle analysis, cells were plated in normal growth media with EGF. After 24 h, the cells were switched to media without EGF and then harvested at various time points to determine whether the cells were growth arrested in G_0/G_1 . For ploidy analysis, tumor cells (passages 6–31) were analysed alone and also mixed with normal primary mouse fibroblast cultures (passage 2). The normal fibroblasts served as a control to establish a diploid mouse histogram.

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